# "TO EVALUATE AND COMPARE THE EFFICACY OF GLYCINE AND CHITOSAN POWDER TO REGAIN THE BIOCOMPATIBILITY OF CONTAMINATED TITANIUM SURFACES: AN IN-VITRO STUDY"

**BY**

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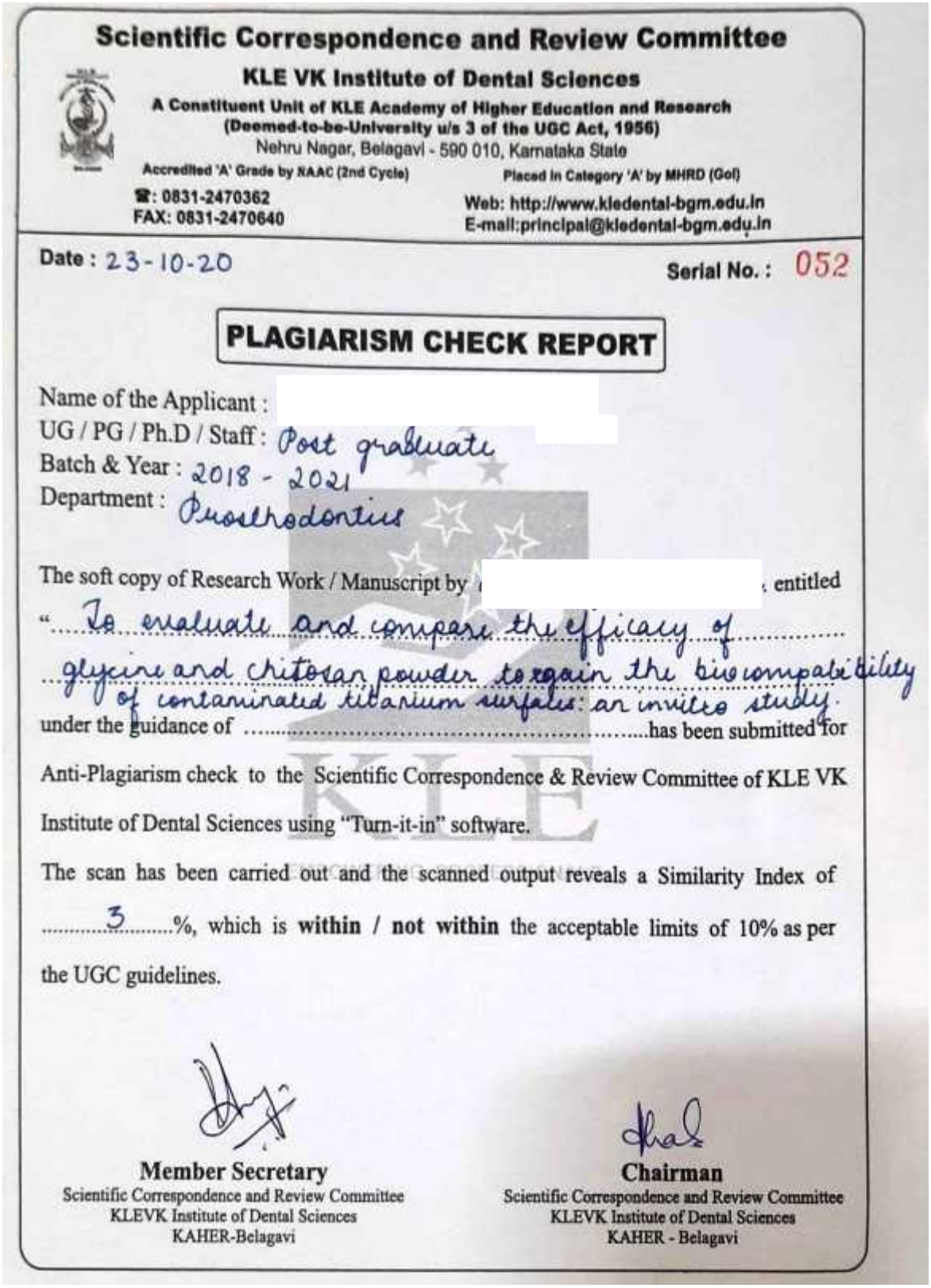

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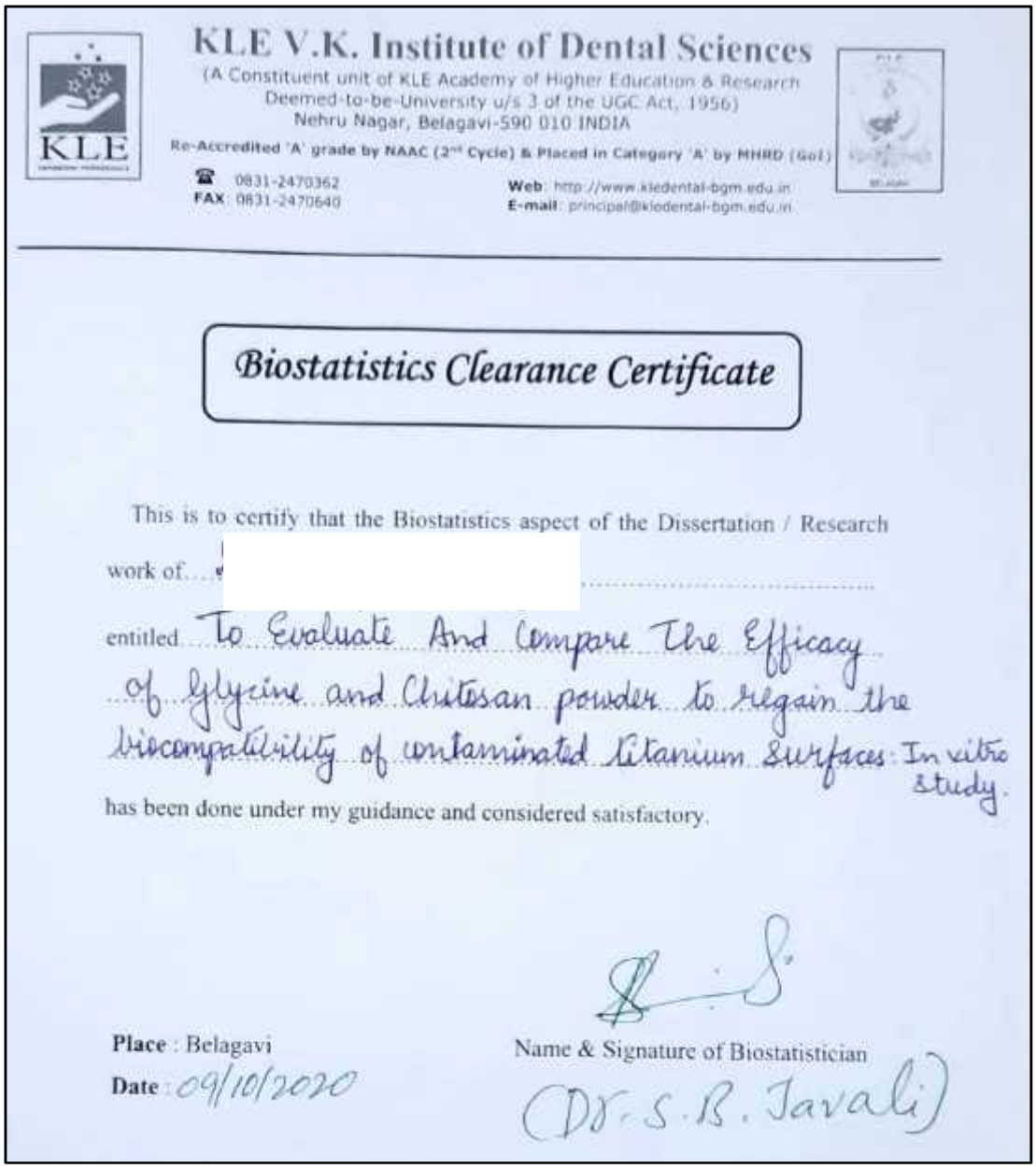

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# LIST OF ABBREVIATIONS USED IN THE STUDY

| | |
|---|---|
| APA | Air Powder Abrasive |
| SLA | Sand Blasted and Acid Etched |
| Group PC | Group Positive Control |
| Group NC | Group Negative Control |
| Group G | Group Glycine |
| Group C | Group Chitosan |
| DMEM | Dulbecco's Modified Eagle Medium |
| FBS | Foetal Bovine Serum |
| BHI | Brain Heart Infusion |
| PBS | Phosphate Buffered Saline |
| DMSO | Dimethyl Sulfoxide |
| CHX | Chlorhexidine |
| AAD | Air Abrasive Device |
| MDA | Mechanical Debridement and Chlorhexidine Solution |
| CAS | Citric Acid Solution |
| TNF-a | Tumour Necrosis Factor - alpha |
| SEM | Scanning Electron Microscopy |
| EDS | Energy Dispersive X-ray Spectroscopy |
| RPA | Residual Plaque Area |
| CFU | Colony Forming Unit |
| WCA | Water Contact Angle |
| HA | Hydroxyl Apaptite |
| TCP | Tri Calcium Phosphate |

| | |
|---|---|
| MIC | Minimum Inhibitory Concentration |
| MBC | Minimum Bactericidal Concentration |
| PI | Plaque Index |
| GI | Gingival Index |
| PPD | Pocket Probing Depth |
| SCAL | Metal Scalar Tip |
| PEEK | Thermoplastic Scalar Tip |
| GLYC | Glycine based Air Powder Abrasive therapy |
| CUR | Curette |
| SOSC | Sonic Scalar |
| DPSC | Dental Pulp Stem Cells |
| ANOVA | Analysis Of Variance |
| SD | Standard Deviation |
| SE | Standard Error |

# ABSTRACT


## STATEMENT OF PROBLEM

Air Powder Abrasive treatment is one of the modality for peri-implantitis. However, there are not many studies describing the role of chitosan as an air abrasive. Chitosan exhibits properties like antibacterial, antifungal, mucoadhesive, osteoconductive and haemostatic properties. Therefore, this study determines the efficacy of chitosan powder to remove the biofilm and decontaminate the titanium surface thereby re-establishing the osseoconductivity of a failing implant surface.

## PURPOSE

The aim of this study is to evaluate and compare the efficacy of Air Powder Abrasive (APA) treatment using Glycine and Chitosan powder in removal of pathogenic biofilm and re-establishing the osseoconductivity of the contaminated titanium surface

## METHODS

Grade 4 titanium discs were pre-contaminated with PorphyromonasGingivalis bacterial strain and stained with crystal violet.APA treatment using water soluble chitosan and glycine powder was carried out. Discs were later washed with ethanol and the absorption of the resultant violet solution was done under a stereomicroscope to evaluate the amount of biofilm removed. Post biofilm removal, discs were disinfected by application of tetracycline and seeded with MG-63 Osteoblast like cells. Cell adhesion was evaluated with haemocytometer and is expressed as the percentage of the initial number of cells. Cell proliferation was assessed by MTT assay with spectrophotometry.

**RESULT**

The collected data was subjected to statistical analysis using One Way Anova and Newman's Kewl Post Hoc Test. There was no significant difference in the removal of biofilm from the titanium surfaces in both the test groups. However, both cell adhesion and proliferation was significantly higher in the group treated with chitosan powder than the glycine powder. ($P < 0.05$)

**CONCLUSION**

Air Powder Abrasive treatment with water soluble chitosan powder can help treat peri-implantitis and thereby re-establish osseoconductivity of infected or contaminated titanium dental implants.

**KEYWORDS**

Air Powder Abrasive, Chitosan powder, Peri-implantitis, Glycine, MTT Assay.

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# INTRODUCTION

Ever since Dr. Per Ingvar Branemark discovered about titanium fusing to bone in 1980, implant dentistry has undergone paradigm shift that has led to the emergence of several new techniques in the field. Dental implantology has changed the way edentulism is being treated. Dental implants have a high success as well as survival rates and thus they can meet the lifelong aesthetic and functional needs of the patient. Patient awareness regarding dental implants has also increased especially in developed countries and up to a certain extent in developing countries also.[1] Due to numerous advantages of implant supported prosthesis; more patients are opting for this treatment modality. According to the American Association of Oral and Maxillofacial Surgeons, 69% of adults aged 35 - 44 have lost at least one permanent tooth due to trauma, periodontal condition, failed RCT or due to tooth decay. 26% will lose all of their permanent teeth by the age of 70 - 80. Almost 1-3 lakhs implants are being placed every year.[2]

Replacing missing teeth using dental implants has a profound positive effect on the overall quality of life of patient. Implant dentistry is predictable with advantages like improved masticatory efficiency, aesthetics, speech, better patient acceptance and an improved sense of self-esteem. Peri-implant tissues consist of soft and hard tissue compartments that are present around osseointegrated dental implants. The function of the peri-implant tissues are as follows: - the mucosa protects the underlining bone, whereas the bone supports the implant. Lack of maintenance and oral hygiene can lead to peri-implant diseases which will hamper the overall success and survival of the implants.[3]

Peri-implant diseases consist of peri-implant mucositis and peri-implantitis. Peri-implant mucositis is concerned with the surrounding peri-implant mucosa and peri-implantitis is concerned with the underlying supporting soft and hard tissue. Peri-implant diseases if left untreated will further cause total failure of the osseo-integrated implant and subsequently the prosthesis. This will further affect the overall health of the patients and cause stress both to the patient as well as the dentist in terms of further treatment plan and financial burden. Peri-implantitis is reversible and can be effectively treated in the initial stages. The process of bacterial infection includes several steps which are bacterial adhesion by initial colonizers, growth and multiplication after attachment of secondary colonizers, and later biofilm formation by a thick layer of exopolysaccharide matrix secreted by the bacterial cells. Once biofilm formation is complete on the implants, the bacterial cells are shielded away from the host immune responses and antimicrobial agents. The antibiotic regimen is generally not effective and thus necessitates removal of the implants, which leads to further expense and discomfort to the patients. [4] Biofilms also prevent the growth or reattachment of osteoblastic cells on the titanium surfaces therefore it is imperative to remove the biofilm so as to re-establish osseoconductivity of the infected titanium surfaces. Different treatment modalities are available ranging from non-invasive procedures like scaling and root planning to surgical interventions namely resective and regenerative procedures.

The Air Powder Abrasive (APA) therapy is one of the mechanical methods used for removal of microbial plaque and deposit present on the implant surface. It was originally developed by Dr. Robert Black in 1945. The APA device propels a pressurized air/water fluid stream containing abrasive particles by kinetic energy through the hand piece nozzle against the tooth surface, thereby removing dental

plaque and stain. The device has a delivery system with two concentric tubes. The central tube propels air along with the abrasive powder particles, while the outer tubes carry the water stream. The water stream coming out from 3 openings act as a shroud and thus limit the dispersion of the air stream, and the two are directed toward the tooth or implant surface on which the polishing slurry of air/powder and water is formed. The cleaning efficiency of APA therapy is high and has resulted in about removal of 85-100% of bacterial endotoxins or biofilms causing minimal or no surface alteration on the implant surface. Cell attachment, viability and proliferation rate post APA therapy has shown to be similar or slightly less compared to sterile specimens within the control. Cellular response is depended on the type, nature and size of the powder used for the treatment. The APA therapy uses different types of powders depending upon supra and sub-gingival scaling.[5]

Biomaterials are non-living materials that interact with the biological system and are used in the medical, biomedical and other fields. Many such materials are used for the replacement of tissues, including bone tissue, as they do not transmit diseases or cause immune rejections, and are available in abundance and at an low cost.[6] Chitosan is one such material which is a deacetylated derivative of chitin. It has a high molecular weight and the second most abundant natural biopolymer mostly found in shells of insects and marine crustaceans and cell walls off fungi.[7] Chitosan has good biocompatibility, is non-toxic to human tissues, biodegradable, selectively permeable, has polyelectrolyte action, antimicrobial activity, ability to form gel, film and sponge, anti-inflammatory action and wound healing properties.. It presents great potential in the repair of bone defects when compared to the limitations of other biomaterials.[5]

Thus, our research evaluates and compares the efficacy of air powder abrasive therapy using water soluble chitosan as the test group and glycine as the control in removal of pathogenic biofilm from titanium surfaces and thereby re-establishing the osseoconductivity for the adhesion and proliferation of osteoblast like cells.

# NEED OF STUDY

Dental implantology has revolutionized dentistry in many ways. Replacement of the lost tissues using the conventional techniques may not be always possible due to complex and challenging clinical conditions. However implant dentistry can overcome this problem even in complex situations like jaw atrophy, compromised health condition etc. Acceptance of osseointegrated implant supported prosthesis has thus increased by the patients.[8]

Titanium and its alloys are the mostly used and preferred biomaterial for manufacturing of dental implants due to its superior biocompatibility; excellent corrosion resistance and superior mechanical properties that make these metals present a mechanical behaviour close to those of bone. Titanium forms a thin layer of titanium oxide which is protective and tightly adhering that prevents its corrosion and makes it biocompatible with the host tissus.[9]

Even though immense progress has been made in the field of implantology, dental implants are susceptible to microbial accumulation and plaque formation which is basically a host associated biofilm. Biofilm on dental implants can cause peri-implantitis, which greatly affects the long-term success of osseointegrated implants and if not treated will cause implant failure. The pathogenic micro-organisms found around an implant with inflammation is similar to those found around natural teeth with periodontitis. The microbial communities within a biofilm environment behave differently compared to the free floating bacteria's. The protective extra cellular matrix makes the bacteria resistant to antibiotics, anti-microbial agents and host defence mechanisms. Mechanical removal is the most effective treatment currently available for the control of dental plaque biofilms.[10]

Many non-surgical and surgical methods are been used for the intervention of the failing implants so as to re-establish the osseoconductivity of the exposed or infected implant surface. These include supragingival and subgingival scaling with carbon, titanium or plastic curettes, air powder abrasive polishing, lasers, local application of antibiotics like chlorhexidine, tetracycline etc. Citric acid or $H_2O_2$ application, photodynamic therapy, resective and regenerative surgery with bone grafts and GTR membranes.[11] However these methods do not ensure complete eradication of the peri-implantitis lesions, therefore a combination of the above treatment options has to be considered in the treatment planning of peri-implantitis.

Air polishing or (APA) therapy is a mechanical method used for removal of the tightly adherent biofilm from the implant surfaces. It uses a pressurized stream of air/powder along with water to remove the deposits present on the surface. Peri-implant mucositis and peri-implantitis can be treated using APA therapy non-surgically and surgically respectively. APA therapy of submucosal implant for treating peri-implant infections is not found to be effective in reducing the inflammation and has less advantage.[12-14] It is difficult to carry out thorough debridement of the bone defect and disinfect the exposed implant surface using APA therapy with a non-surgical approach. Achieving re-osseointegration in such case thus becomes a hurdle.[15] Thus, it is imperative that APA therapy should be used in a surgical approach which can thus lead to the improvement of the periodontal clinical parameters thereby combatting peri-implant infections effectively. [16]

Many chemical agents like citric acid, tetracycline HCl, chlorhexidine gluconate, hydrogen peroxide have been used for dental implant detoxification in the treatment of peri-implant diseases.[12] APA therapy was found to be more advantageous

when compared to these chemical agents. The powders which are generally used for APA treatment include hydroxyapatite, tri-calcium phosphate, glycine, sodium bicarbonate, titanium dioxide etc.[11]

However there are no studies evaluating the use of chitosan in a powder form to be used for the APA therapy. Chitosan is a versatile and quite unique bio-based polymer. It is derived from chitin which is commonly found in invertebrates like crustaceans or insect cuticles. This polysaccharide i.e. chitosan has a remarkable antimicrobial activity as it can alter the cell permeability and to bind with the cellular DNA which inhibits the RNA synthesis. It is also biodegradable to non-toxic residues because of the presence of glycosidic bonds within the polymer. It also demonstrates haemostatic activity because of the interaction of positively charged chitosan molecules with the red blood cells that are negatively charged. It has also be used for preparation of biomaterials which are used as a substitute for missing tissue or organ, and allow cell attachment and proliferation. Some of the other desirable properties of chitosan are anti-fungal and anti-inflammatory activity, mucoadhesiveness, and its ability to increase the proliferation of osteoblasts.

Therefore keeping in mind the benefits of this unique material, it is imperative to find its usage with regards to the perennial problem of peri-implantitis. Therefore, this in-vitro study is concerned with APA therapy using water soluble chitosan as the test group and glycine as the control group in treatment of peri-implantitis.

# HYPOTHESIS

**NULL HYPOTHESIS:**

There is no significant difference in removal of the pathogenic biofilm and the cell proliferation and adhesion when treated with Chitosan and Glycine Powder using Air Powder Abrasive Therapy.

**RESEARCH HYPOTHESIS:**

There is a significant difference in removal of the pathogenic biofilm and the cell proliferation and adhesion when treated with Chitosan and Glycine Powder using Air Powder Abrasive Therapy.

# AIM AND OBJECTIVES

## AIM OF THE STUDY:

Aim of the study is to evaluate and compare the efficacy of Glycine and Chitosan powder in removal of pathogenic biofilm using Air Powder Abrasive therapy and re-establishing the biocompatibility of the contaminated titanium surface.

## OBJECTIVES:

- To evaluate and compare the efficacy of glycine and chitosan powder in removal of the pathogenic biofilm using air powder abrasive therapy and reestablish the biocompatibility of contaminated titanium surface.
- To evaluate and compare the cell proliferation and cell adhesion of MG-63 osteoblast like cells after air powder abrasive therapy using glycine and chitosan powder.

# REVIEW OF LITERATURE

1. **Dennison (1994)** studied the relationship between the implant surfaces and disinfection methods. The study evaluated the most effective treatment and the implant surfaces that were most effectively decontaminated. Press fit cylindrical specimens of titanium with plasma sprayed, machined, and hydroxyapatite-coated surfaces were used for the study. Endotoxin was prepared from P.gingivalis. 125I-LPS was coated on the implants and then treated by - burnishing it with cotton pellet soaked in water, 0.12% Chlorhexidine (CX), citric acid solution (CAS), or treated with APA therapy (AIR). Radioactivity was evaluated after 2 treatment cycles of each. Remaining 125I-LPS after two treatment cycles for each group were: machined implants - AIR < CA; for plasma sprayed implants AIR<water; for hydroxyapatite implants AIR = CA <water<CHX. Machined implants were disinfected more effectively than other surfaces compared to other treatments however citric acid treatment was equally effective on both machined or hydroxyapatite surfaces thus being an exception. According to the study, machined implants without any surface coatings are decontaminated by a many methods. Therefore, such characteristic can be reasoned, as long term success of implants involves treating periimplantitis. Also, the study indicates that APA therapy is effective for disinfecting implant surfaces.[17]

2. **Matthias Kreisler (2003) e**valuated and compared the biocompatibility and disinfection of titanium discs infected with Porphyromonas Gingivalis after APA and ER:YAG laser treatment. Proliferation of the gingival fibroblast cells was evaluated by Alamar Blue Assay and the cells were seeded on titanium

discs after the disinfection. Proliferation was less on the control specimens and it was not significantly different in the APA and ER:YAG laser group compared to the sterile specimens. Microscopic surface changes were seen on the discs treated with APA therapy and no changes were seen on the discs treated with ER:YAG laser. Both the disinfecting methods proved to have better potential in removing bacterial components and re-establishing biocompatibility for cellular growth.[18]

3. **Maximo (2009)** evaluated the microbiological and clinical outcome of anti-infective therapies for peri-implant infections. Patients having dental implants were assigned to healthy, mucositis or peri-implantitis groups. Implants with peri-implant infections were disinfected by Teflon curettes and air-powder abrasive therapy using sodium carbonate, performed surgically for peri-implantitis and without surgery for mucositis. Periodontal clinical parameters were evaluated at baseline and at 3 months after treatment. Submucosal plaque samples were collected and analysed for 40 bacterial species. Clinical parameters improved at 3 months after therapy in patients with mucositis and peri-implantitis. The mean reduction in relative clinical attachment level was 1.4 mm and 2.3 mm, and for probing depth it was 1.3 mm and 3.1 mm for mucositis and peri-implantitis, respectively. Levels of Tanerella forsythia, Treponema denticola and Fusobacterium nucleatum reduced after peri-implantitis and mucositis therapy, respectively. Counts of Treponema socranskii, Porphyromons gingivalis and bacteria belonging to the red complex were reduced in both groups at 3 months after treatments. Mechanical therapies were efficacious in treating mucositis and periimplantitis over a period of 3

months. The surgical procedure proved to be beneficial for treating peri-implantitis and can be used as a standard control for future studies.[19]

4. **Mendonca (2009)** presented a case series in which the effects of anti-infective mechanical therapy for peri-implant infections was evaluated on the clinical parameters and levels of tumor necrosis factor-alpha (TNF-a) after 12 months. Ten patients having peri-implantitis were subjected to open surgical disinfection using APA treatment using sodium carbonate and resin curettes. Clinical parameters were recorded at baseline and 3 and 12 months after the therapy. TNF-a in the peri-implant crevicular fluid was measured using enzyme-linked immunosorbent assay. Improvements were observed after the anti-infective therapy in clinical parameters at 3 and 12 months. Changes in mean bleeding on probing and probing depth had also significantly reduced. Levels of TNF-a in the crevicular fluid in sites affected by peri-implantitis had reduced after anti-infective mechanical therapy over a period of 12 months which improved the clinical condition of the patients.[20]

5. **Sahm N (2011)** evaluated and compared the efficacy of air-abrasive device (AAD) with mechanical debridement with carbon curettes and anti-septic therapy with chlorhexidine digluconate (MDA) along with oral hygiene program for both the groups for non-surgical treatment of peri-implantitis. Periodontal clinical parameters were measured at baseline, 3 and 6 months after treatment. Patients receiving Air abrasive device therapy (AAD) presented with higher reductions in the bleeding on probing scores compared to the MDA group. However, there were no significant differences in the probing depth reduction and gain in clinical attachment level in between both the groups.[21]

6. **Ceylin S. Tastepe (2012)** used APA treatment with calcium phosphate for disinfection of titanium discs which were contaminated intraorally. Sand blasted and acid etched (SLA) titanium discs were kept intraorally for 48 hours and then stained with erythrosine dye to make the biofilms visibl.th discs were divided into 6 groups and APA treatment was carried out by using following materials:

    1) Water and air (Control).
    2) Hydroxyl apatite (HA).
    3) Hydroxyl apatite along with Calcium Phosphate (HA + TCP).
    4) Titanium Dioxide (TiO2).
    5) EMS Soft Sub gingival powder (EMS)
    6) Phosphoric Acid.

    Surface changes, surface chemical content and Residual biofilm were evaluated with Scanning Electron Microscopy (SEM) and Energy Dispersive X-Ray Spectroscopy (EDS). All the groups showed reduction in the biofilm content except for phosphoric acid group. The highest biofilm removal was seen in HA+TCP group (99%) followed by the HA and MS group. Surface structure had minimal changes after the treatment was carried out. APA treatment using bioactive and osteoconcoductive powder can be used as an effective treatment for removal of biofilms. Surface topography of titanium is preserved and attachment of bioactive powder particles are seen on the surface.[22]

7. **Sahrmann (2012)** studied the accessibility of an Air Powder Abrasive therapy using glycine powder to implant surfaces in in-vitro models simulating various types of peri-implantitis defects. Eighteen implants were coated with a red colour ink and placed in different defect morphologies. Vertical bone

angulations of 15°, 30°, 60° and 95° were prepared and the implants were treated with APA therapy using glycine powder. Photographs of the implants were captured in three directions i.e. perpendicular to the implant axis and at an angulation of 60° and 120°. The area with residual colour represented areas that were inaccessible to the powder. Percentages of area with residual ink were calculated. The median percentages of uncleaned areas in the 15°, 30°, 60° and 90° defects were of 51%, 24%, 8%, and 3%, respectively. The coronal third was better disinfected than the apical third. This finding prominent in narrower defects. Complete removal of the residual ink from the implant surfaces could not be performed in any of the defects but most of the surface could be decontaminated in larger defects. In steep angular defects and below the threads, residual stained areas remained on the implant surfaces.[23]

8. **Ying-jie (2013)** conducted a pilot study that evaluated the effect of APA treatment using glycine powder for treatment of peri-implant mucositis. Twenty-four patients having at least 1 implant with peri-implant mucositis were sorted into test and control groups. Sites with probing depth more than 4 mm were treated by APA therapy using glycine for 5 secs in the test group. Clinical parameters were measured at 1-week, 1-month, and 3-months. The mean reductions in probing depth at site level were 0.93 mm and 0.91 mm in the test and control groups, respectively at the end of 3 months and no significant difference were present between the two groups. The pilot study suggests that adjunctive APA treatment using glycine had a limited beneficial effect compared to mechanical debridement alone.[24]

9. **Drago (2014)** used a new formulation which consisted of chlorhexidine and erythritol and compared it with glycine powder used in APA devices. Their in vitro efficacy to decontaminate Staphylococcus aureus, Bacteroides fragilis, and Candida albicans were investigated. Biofilm was grown on SLA titanium disks and were subjected to APA therapy with glycine and erythritol-chlorhexidine powders. Spectrophotometric assay was performed for semiquantitative analysis of the biofilm. Confocal laser scanning microscopy was performed for qualitative analysis. Minimum inhibitory concentrations (MIC) and minimum microbicidal concentrations (MBC) were evaluated, after air-polishing treatment. The chlorhexidine - erythritol combination demonstrated stronger antimicrobial and antibiofilm activity compared to glycine against all microorganisms tested. Air Powder Abrasive therapy with chlorhexidine - erythritol proved to be a better alternative to the conventional glycine based treatment for biofilm removal.[25]

10. **Toma (2014)** analysed the effect of an air-abrasive device for surgical treatment of peri-implant infections. Implants with peri-implantitis were surgically treated using an APA device (Perio-Flow), or plastic curettes and cotton pellets soaked with saline (control group). Periodontal clinical parameters and radiographs were previously assessed at baseline, 6 months, and 12 months after treatment. Plaque Index (PI) scores remained low during the observation period for all the groups. At the end of the study, Gingival Index (GI) and Probing Pocket Depth (PPD) reductions were greater in the Perio-Flow group. Bone Loss levels showed no differences between baseline and 12 months. Within the limitations of the study, both groups i.e. Perio-Flow and control group demonstrated a significant reduction of the clinical parameters .[26]

11. **Vincent Bennani (2015)** evaluated biofilm removal, biocompatibility and morphological changes due to glycine powder based APA therapy on titanium surfaces. Grade IV titanium surfaces were subjected to S.Mutans biofilm formation. Electron-dispersive spectroscopy (EDS), scanning electron microscopy (SEM) and confocal microscopy were carried out to assess the characteristics of the disc. A crystal violet dye-binding assay was used to quantify bacterial biofilms. L929 fibroblast cells were cultured on the discs and their coverage and viability was measured. Residual biofilm on discs receiving treatment was lower than untreated controls. Both groups had similar cell viability but coverage by fibroblasts on treated discs was half that on untreated discs. Air-polishing removed considerable amount of biofilm from titanium surfaces.[27]

12. **Gordon John (2015)** evaluated the efficacy of APA treatment using tricalcium phosphate and glycine in removal of biofilm from titanium and zirconium oxide surfaces. Plaque was collected intraorally on titanium and zirconium oxide specimens which were worn on a splint to form the biofilm. The specimens were randomized to test groups which used the glycine and tricalcium phosphate powder whereas the control group used the sodium bicarbonate or glycine powder. Residual plaque areas (RPA) were measured as percentage of the whole measuring field. The specimens were then seeded with SAOS-2 cells which have the osteoblast like properties to evaluate biocompatibility of treated surfaces. Cell viability was measured by using luminescence assay in a luminometer. Scanning electron microscopy and energy dispersive X-ray microanalysis was used to assess the change in surface morphology. Mean RPA for the titanium discs was the highest with sodium bicarbonate followed by

glycine and tricalcium phosphate + glycine. Mean RPA for the zirconium oxide discs was highest for glycine followed by sodium bicarbonate and tricalcium phosphate + glycine. Therefore tricalcium phosphate + glycine seemed more effective for biofilm removal on titanium and zirconium implant surfaces than the control groups.[28]

13. **Lombardo (2015)** used a novel approach for treating peri-implantitis by using a topical desiccant i.e. HYBENX and APA therapy as a mean of disinfection, followed by the application of biphasic calcium sulphate combined with inorganic bovine bone material to augment intrabony defect. A 62-year-old man with periimplantitis at two neighbouring implants in positions 13 and 12 underwent flap surgery, followed by the novel procedure. Both implants revealed an absence of bleeding on probing, regeneration of missing bone, and reduction in pocket probing depth along with clinical attachment gain after a period of 2 years. Slight mucosal recession was observed but there was no reduction in keratinized tissue. Based on the results, use of HYBENX and APA therapy, followed by bone grafting, seems to be a viable option for treating peri-implantitis.[29]

14. **Chen (2016)** conducted a study to evaluate the potency of three decontamination methods for bacterial removal from titanium surfaces and bacterial adhesion on titanium implant having different surface roughness. Titanium samples were divided into 5 groups: No. 1200 grit sandpaper polishing (Grit), 50 µm (SB50), 100 µm (SB100), and 250 µm Al2O3 sandblasting (SB250), and sandblasting, large-grit, and acid-etching (SLA). Surface topography was examined. Adhesion of E.coli on different surfaces was

assayed. After incubating the bacterial culture for 8 h, three cleaning treatments, including plastic curettes, APA therapy, and Er:YAG laser, were used on the specimens. There was no difference in the adhesion of E.coli among all the roughened groups, except the SB50 and SB250 groups at 12 h of culture. No significant surface alterations were found after use of three cleaning treatments. E. coli adhesion was reduced aftr using air-powder abrasive and laser treatment in compared to the samples treated with plastic curettes. To conclude, laser debridement can be an effective cleaning method for peri-implantitis.[30]

15. **S M Lupi (2016)** studied the efficacy of APA therapy using glycine powder for the maintenance of peri-implant health status in patients receiving implant supported prosthesis and compared it to manual debridement and chlorexidine administration treatment group (MDA). Periodontal clinical parameters were analysed. Clinical data was collected before treatment and at 3 and 6 months after the treatment. Treatment with APA showed considerable improvement in the bleeding and plaque index, probing depth, and bleeding score. There was no change in clinical attachment level in both the groups. APA therapy using glycine powder was more effective than manual debridement with plastic curettes and chlorhexidine in maintenance of the peri-implant health.[31]

16. **Ronay (2017)** simulated non-surgical peri-implantitis treatment in an in-vitro model to evaluate the efficacy of air powder abrasive therapy and compared to ultrasonic scaling and manual scaling with curettes. Dental implants were ink-dyed with ink and mounted in a soft and hard tissue model in vitro model, simulating peri-implantitis defects with angulations of 30, 60, and 90° covered

by an artificial mucosa. Ultrasonic scaler, Gracey curette and an APA device using glycine powder was used for the debridement procedures. Implant surface images were obtained. Areas with colour remnants were determined and their average was calculated. Change in the surface was analysed on scanning electron microscope (SEM) images. The unclean areas for ultrasonic tips, curettes and APA accounted for 66.95 ± 8.69%, 74.70 ± 4.89% and 33.87 ± 12.59% respectively. The APA device showed better results for all defect angulations. Surface alterations were seen after instrumentation with Gracey curettes and ultrasonic devices in SEM images, whereas glycine powder did not display any surface alterations. The APA device proved to be superior decontaminating method for all defect angulations.[32]

17. **Al Ghazal (2017)** conducted a pilot study to compare the efficacy of two different methods of disinfection to maintain and improve peri-implant soft tissue health over a period of 12 months. Twenty adult patients, in whom 25 implants were placed, volunteered in a randomized clinical trial. Signs of pathologic bone loss were not found in any of the implant. Patients were reviewed every 3 months over a period of 12 months. Nine patients in which 15 implants were placed were randomly allocated to a test group and treated with APA therapy and another nine patints in which 10 implants were placed, were allocated to a control group and treated with titanium curettes. Peri-implant crevicular fluid was analysed to quantitatively measure the concentration of six interleukins. No differences were to be found in bleeding on probing (BOP) between the two treatment methods. Both disinfection methods demonstrated similar reduction of BOP. Both treatment modalities were effective in reducing peri-implant inflammation.[33]

18. **Stein (2017)** aimed to study the clinical outcomes of stepwise mechanical debridement combined with Povidone-iodine application with and without systemic antibiotics. Patients with chronic periodontitis including 164 screw-typed implants with periimplantitis were selected for the study. Ultrasonic debridement, soft tissue curettage, glycine powder air polishing and a repeated submucosal application of Povidone-iodine was carried out for all the implants. In cases with severe periodontitis, amoxicillin and metronidazole were prescribed for 7 days. Implants treated without Amoxicillin and Metronidazole showed significant reductions changes of mean probing depth, clinical attachment loss and bleeding on probing were more pronounced after a period of 12 months. Intake of above mentioned antibiotics did not influence the changes of these parameters. However, the reduction of implant sites with PD >4 mm and BOP was greater in patients with antibiotics than in those without. The combination of ultrasonic scaling, soft tissue curettage and glycine air polishing with adjuvant Povidone-iodine led to clinical improvements at implants.[34]

19. **Govindharajulu (2017)** developed an osseoinductive coating developed with the combination of chitosan and elastin-like biopolymer (P-HAP). Chitosan/P-HAP bi-layers were coated on the titanium surfaces by layer-by-layer assembly method which was evaluated with X-ray photoelectron spectroscopy and atomic force microscopy. Significant growth of mouse pre-osteoblast and biomineralization was seen on the titanium surfaces with layer by layer deposition of the chitosan/P-HAP along with adequate antibacterial activity against Streptococcus Gordonii.[35]

20. **Larsen (2017)** carried out the disinfection of dental implants contaminated with virulent and avirulent strains of P.Gingivalis using three different instrumentations. Dental implants were divided into three groups based on the method of decontamination which was: - 1) ER: YAG laser 2) chitosan brush and 3) titanium curettes. Checkerboard DNA-DNA hybridization was used to evaluate the anti-microbial effect of the decontamination method. Light interferometer was used to check the implant surface alterations. Adhesion of P.Gingivalis was reduced significantly in all three groups. Er:YAG laser proved to be effective in elimination of both bacterial strains. The titanium curette significantly altered the implant surface micro-texture. Chitosan brush and Er:YAG laser did not alter the implant surface. All the treatment modalities appear to have a similar potential to against P. gingivalis.[36]

21. **Rutger Matthes (2017)** investigated the effects of biofilm removal from titanium discs with an APA and a cold plasma device on cells in vitro. APA device using erythritol powder (AP), cold atmospheric plasma (CAP) or a combination of both (CAP + AP) was used for elimination of biofilm. MG-63 Osteoblast-like cells were then seeded onto the titanium discs to evaluate the biocompatibility of the titanium surfaces. Scanning electron microscopy was used to evaluate the cell viability of MG-63 cells. Surface hydrophilicity was analysed by measuring the water contact angle (WCA) of the disc for each treatment method. 85% of the titanium surface treated with AP was covered with cells and those treated by combination of AP+CAP ranged from 57% up to 75%. For the CAP treatment group, microorganisms were not effectively removed and destroyed all cells. All the treatment methods reduced the water contact angle. AP treatment proved to be efficient in removing biofilm from

rough implant surfaces when compared to the combination of plasma and air-polishing treatment or CAP alone.[37]

22. **David George Quintero (2017)** conducted an in-vitro study to assess the potency of APA therapy on a novel peri-implantitis defect model. 26 implants were inoculated with S.Sanguinis biofilm. 6 implants served as the positive and negative controls and the remaining 20 implants were treated either with Cavitron Jet Plus and Air-Flow Perio. Residual bacteria were cultured and the CFU's were measured. The negative controls had no presence of bacteria whereas the positive control had a CFU per streak of 104. Specimens treated with the APA therapy had a reduction of 99% of bacterial counts with CFU of 10 and 4 for Cavitron JET Plus and AIR-FLOW PERIO.[38]

23. **J. C. Wohlfahrt (2017)** assessed chitosan brush placed in a dental hand piece for non-surgical treatment of mild peri-implantitis and evaluated the effect on peri-implant mucosa. Subjects with mild peri-implantitis were clinically examined at baseline and after 2, 4, 12 and 24 weeks. Radiographs were taken at baseline and at 3 and 6 months. Implants were treated with chitosan brush placed in a dental hand piece at baseline and at 3 months. Significant reductions in both pocket probing depth and bleeding on probing were observed at all intervals compared to baseline clinical measurements. The mean pocket probing depth and bleeding on probing at baseline were 5.15 mm and 1.86, respectively. At 6 months the values for the same were 4.0 mm and 0.64, respectively. Stable reductions were evident in both the parameters up to 6 months after the initial treatment and 3 months after the second treatment. Stable radiographic levels of osseous support were present in all the treated implants. The study demonstrated

that a chitosan brush is safe to use and is effective in the non-surgical treatment of dental implants with mild peri-implantitis.[39]

24. **Magda Mensi (2018)** evaluated plaque removal and the preventive efficacy of erythritol/chlorhexidine APA powder and compared it with sodium bicarbonate. AIR-FLOW HANDY 3.0 PERIO system was used to carry out APA treatment. Mechanical profilometry was used to assess the abrasiveness of the powder by evaluating the surface roughness of the titanium discs by profilometry before and after APA treatment. Titanium discs were subjected to APA treatment using both the powders and then submerged in fresh bacterial culture of either Staphylococcus aureus or Aggregatibacter actinomycetemcomitans. The discs were incubated for 24 h and number and viability of bacteria's were evaluated based on CFU count and colorimetric XTT assay. Plaque removal was also evaluated after subjecting infected titanium discs to APA treatment and bacterial cell viability was evaluated. APA treatment with both the powders showed reduction in the bacterial biofilm viability of the pre-infected specimens whereas only Erythritol-CHX powder was effective in reducing the bacterial biofilm viability in the pre-treated titanium discs. Profilometry showed no surface changes on the titanium discs regardless of the powder used.[40]

25. **He (2018)** in his study, prepared a gelatin (G) and chitosan (C) composite GBR membrane containing hydroxyapatite nanoparticles (nHA) and antimicrobial peptide (Pac-525)-loaded PLGA microspheres (AMP@PLGA-MS), by sequential layer-by-layer electrospraying and electrospinning techniques. Ideal biocompatibility with good cell adhesion, proliferation and spreading was observed with in vitro cell culture of rat bone marrow mesenchymal stem cells

(rBMSCs). Osteogenic differentiation of rBMSCs was observed with the G/C membrane containing nHA was used. The composite GBR membrane containing AMP@PLGA-MS displayed a long-term sustained release of Pac-525, which had antibacterial activity against S. aureus and E. coli. According to the study, antimicrobial peptide-loaded G/C composite membrane can be used in future for guided bone augmentation procedures with anti-bacterial activity.[41]

26. **Rosen (2018)** performed a study to determine whether mechanical debridement, followed by APA therapy with glycine, followed by application of citric acid with cleaning the surface with sterile water after each step, is efficient in cleaning an infected implant surface. Implants that were considered hopeless due to advanced peri-implantitis were extracted. They were divided into three groups. The test group were exposed to the decontamination protocol; the control group was untreated or mechanically treated, followed by rubbing the surface with sterile saline. All implants were placed in culture with human osteoprogenitor cells for 72 hours. Scanning electron microscopy was performed for evaluation. The test implants demonstrated cell adhesion and proliferation of the normal human osteoprogenitor cells on previously contaminated surface. Cell attachment and proliferation was not observed on the untreated implants. Therefore, this protocol is effective for disinfection of implants with peri-implantitis.[42]

27. **Anastasiou (2019)** synthesised fluorapatite and doped it with $Sr^{2+}$ and $Ce^{3+}$ ions. Undoped and $Ce^{3+}$ doped fluorapatites exhibited better antibacterial response than the $Sr^{2+}$ doped material against bacterias like S. Aureus, B. Subtilis, E. Coli, B. Cereus. Chitosan scaffolds were incorporated with the

synthesised minerals and tested it with Dental Pulp Stem Cells (DPSC) to study their regenerative potential. $Sr^{2+}$-doped fluorapatite scaffolds, showed greater osteoconductivity leading to the differentiation of the DPSC's into osteoblasts. Percentage of osteocalcin and the RUNX2 gene expression were high compared to the un-doped mineral when $Ce^{3+}$-doped material was used. The conducted research suggests that antibacterial properties of fluorapatite were retained when doping was carried out with $Ce^{3+}$ and increases its regenerative potential, making it a valid option for dealing with conditions where healing of hard tissues is compromised by bacterial contamination.[43]

28. **Cha (2019)** evaluated the implant surface topography and roughness of implants after using five decontamination methods in an in-vitro study. Sandblasted and acid-etched (SLA) implants were placed supra-crestally. Implants were subjected to one of the following methods: a metal scaler tip (SCAL), a thermoplastic scaler tip (PEEK), a round titanium brush (RBRU), a tufted brush with titanium bristles (TNBRU), and a glycine-based air-powder abrasive (GLYC). The sixth group was considered as the control. Implant surface topography was examined using SEM and CLSM. SCAL lead to macroscopic change and damage to the implant surface, remnants of the plastic tip were left on the surface when PEEK was used and both titanium brush groups flattened the thread profile were flattened when the titanium brush was used, and GLYC showed the least change in the surface. Within the limitations of this in vitro investigation, the tested protocols induced different macroscopic alterations and surface roughness changes that varied in the thread and valley area.[44]

29. **David Keim (2019)** conducted a study that evaluated the efficacy of different implant surface decontamination methods in an in-vitro peri-implant bone defect model. Dental implants were stained using red coloured dye and were divided into 3 groups of a standardized peri-implant bone defect resin models with a surrounding defect angulation of 30°, 60°, or 90°. Implants were allocated to each type of defect and were sub-divided to one of the three decontamination method which was: 1) curette (CUR), APA with glycine powder (APA) or sonicscaler (SOSC). Average of surface area not decontaminated was measured by colour recognition technique after photographs were taken of each implant from both sides. Morphologic surface damages were assessed by Scanning electron micrographs (SEM). The percentage of residual colour for each decontamination procedure in all three defect angulations was significantly different. The values for each group were as follows were as follows :- 30° group CUR: 53.44% > SOSC: 19.69% > APA: 8.03%; 60° CUR: 57.13% > SOSC: 11.4% > APA: 0.13%; and 90° CUR: 48.1% > SOSC: 13.07% > APA: 0.58%. APA treatment modality was effective compared to curettes and sonicscaler and caused less surface damage.[45]

30. **Victor H. Matsubara (2019)** investigated the efficacy of various powders using APA therapy to remove biofilm and their effect on titanium implant surfaces. Twenty implants were coated with permanent red ink and inserted into 3-D printed circumferential bone defect models. APA treatment was carried out with three powders namely sodium bicarbonate, glycine, and erythritol. Water was used as the control. Digital photography and graphic software was used to assess the % of remaining ink. Optical profilometry was used to quantify implant surface topography and surface roughness and examined via scanning

electron microscopy. The microscopic analysis was performed at the collar (Laser-Lok surface) and threads of the implants. The percentage of cleaned surfaces after treatment with Sodium bicarbonate, erythritol and glycine was 49.3 ± 3.6%, 25.1 ± 0.7%, and 33.1 ± 1.2%, respectively. Sodium Bicarbonate increased the implant roughness on implant collar and threads. Glycine and erythritol showed no changes in surface roughness but limited ink removal capacity was demonstrated.[46]

31. **Seong-Ho Jin (2019)** aimed to evaluate the biocompatibility of titanium disks treated with several decontamination methods. Salivary bacterial contamination with dental pellicle formation was used as an *in vitro* model. SLA titanium disks were used. Three control groups made were 1) pristine disks (SA group); salivary pellicle-coated disks (pellicle group); and biofilm-coated, untreated disks (NT group). Decontamination of the biofilm-coated disks was carried out by 14 methods. MG63 cells were cultured on the treated disks. Cell proliferation assays were performed, and cell morphology was analysed by immunofluorescence and scanning electron microscopy (SEM). VEGF assay was performed on day 5 of culture. Cell proliferation assay demonstrated that all decontaminated disks showed significantly less cell proliferation than the SA group, except for the 2 groups treated using a plastic tip. Most groups demonstrated adequate cell density, with the exception of the NT group, in which the cell density was less and bacterial residue was observed. Lower VEGF production was observed than those in the SA group on tetracycline-treated titanium disks. No decontamination method proved to be completely efficient in restoring the biocompatibility similar to that of the sterile or pristine discs. However, they lead to improvement in the biocompatibility of the

titanium disks when compared to the biofilm-coated and untreated titanium disks. Thus, decontamination is of utmost importance for the treatment of peri-implantitis.[47]

32. **Koldsland (2020)** assessed the effect of titanium curettes and chitosan brushes following a surgical treatment of periimplantitis when performed every third month from six to 18 months. Patients were randomized into two groups based on the peri-implant treatment i.e.by using titanium curettes or chitosan brushes on implants. Follow-up examinations and supportive therapy was performed every three months post surgically. The number of implants recorded with inflammation was high at the 6-month baseline examination in both, the test as well as the control group and remained high throughout the observation period. Similar observations were made for all clinical parameters and no differences were found between test and control groups. Therefore in the present study, titanium curettes or chitosan brushes were equally effective in treatment of peri-implantitis.[48]

33. **Tuchscheerer (2020)** evaluated in vitro efficacy of surgical and non-surgical APA treatment for implant surface disinfection. 180 implants were allocated to three different angulated bone defect models (30°, 60°and 90°). Biofilm was made using indelible red colour. 60 implants were used for each defect, 20 of which subjected to APA therapy with 3 different types of glycine air powder abrasion combinations. A surgical and non-surgical procedure was simulated within 20 equally air-polished implants. All implants were photographed to determine the uncleaned surface. Scanning electron micrographs (SEM) were used to evaluate changes in surface morphology. No differences were seen

between GAPA1–3 for surgical and non-surgical application. Notable differences were observed for GAPA2 between 30° and 90° in the non-surgical and 30° and 60° in the surgical simulation. The surgical use of APA was superior to the non-surgical. SEM micrographs displayed no surface damages after use of Glycine APA. APA therapy is an efficient, protective method for surgical and non-surgical implant surface disinfection in this In-vitro model. None of the methods were sufficiently effective in decontamination protocol[49]

34. **Lollobrigida (2020)** aimed to study four different treatment methods on titanium surfaces with moderately rough surface. APA therapy (APA) using glycine powder, a diode laser at 3 W (L3) and 4 W (L4) and a titanium brush (TB) were used. Surface roughness, morphology and chemical composition of the titanium surfaces were analysed by white light interferometer, SEM and X-ray photoelectron spectroscopy (XPS), respectively. Significant alterations in surface morphology on TB samples were observed, while AP and L3 had only a lesser impact. L4 samples displayed signs of overheating. Surface roughness and surface chemical composition was not altered. APA therapy using glycine powder and 3 W diode laser caused lowest impact on the physicochemical properties.[50]

# MATERIALS AND METHODS

**SOURCE OF DATA:**

This in-vitro study was performed in the Department of Prosthodontics and Crown and Bridge, KAHER Vishwanath Katti Institute of Dental Sciences, Belagavi and Dr. Prabhakar Kore's Basic Science Research Center, KAHER, Belagavi and Department of Pharmaceutics, KLE College of Pharmacy, Belagavi.

**SAMPLE SIZE:**

A total of 144 sandblasted and acid etched (SLA) grade 4 titanium discs were used for the study which were divided into four groups of 36 samples namely :-

1) Group PC :- Discs with biofilm and not exposed to any treatment. (Positive Control)
2) Group NC :- Sterile discs without any biofilm. (Negative Control)
3) Group G :- Discs with biofilm and treated with APA therapy using Glycine.
4) Group C :- Discs with biofilm and treated with Water Soluble Chitosan.

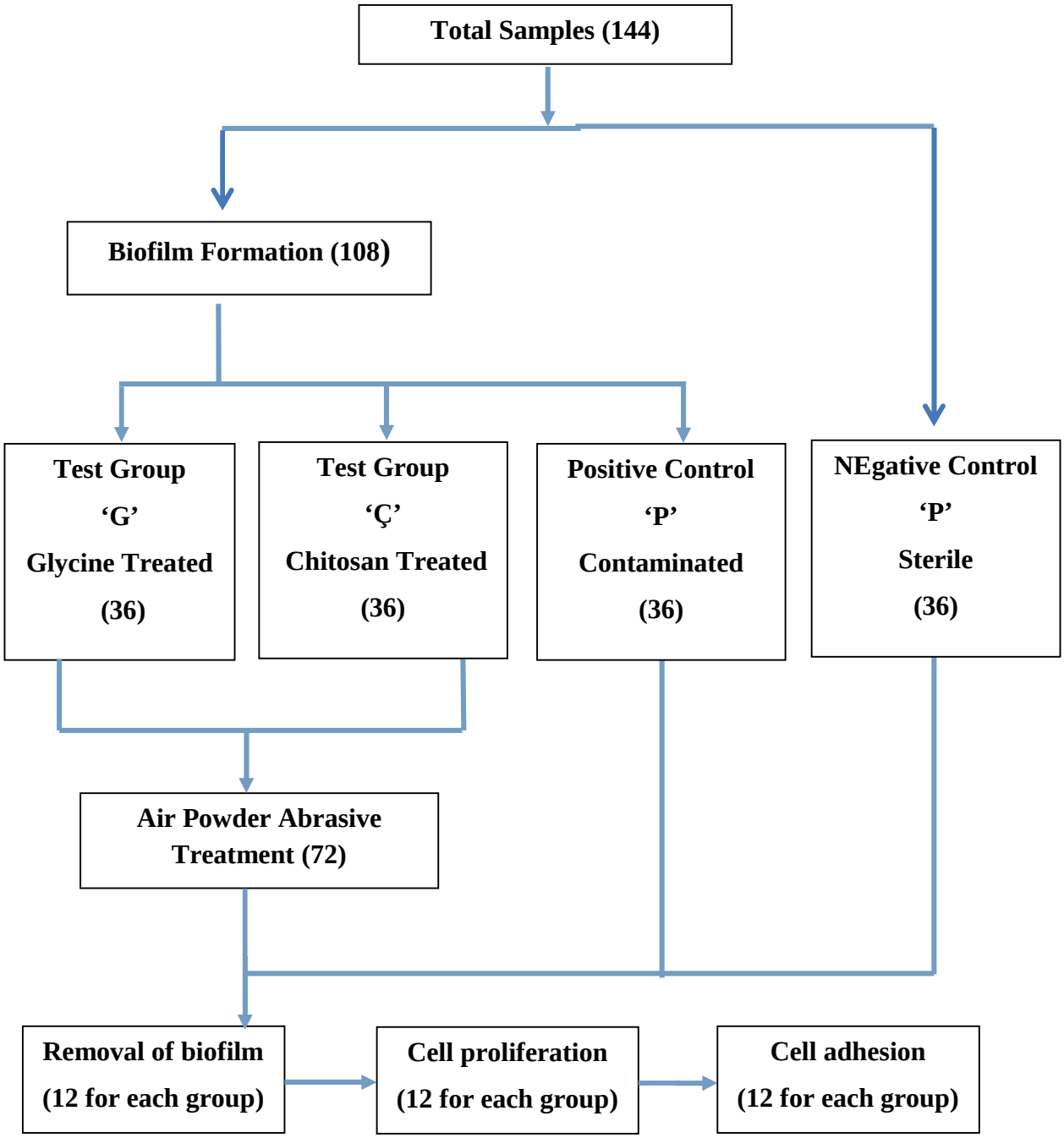
Each group was further divided into 3 sub-groups of 12 for evaluation of:-

A) Biofilm Removal

B) Cell Proliferation

C) Cell Adhesion

**SCHEMATIC REPRESENTATION OF SAMPLE SIZE:**



**INCLUSION CRITERIA:**

- Identical titanium discs.
- Titanium discs with identical size and shape.

**EXCLUSION CRITERIA:**

- Titanium discs with surface defects and deformities.
- Titanium discs without biofilm formation.
- Contaminated equipment's and materials.

**TABLE 1: MATERIALS USED IN THE STUDY**

| MATERIAL | MANUFACTURER |
|---|---|
| Grade 4 sandblasted acid etched Titanium discs. | Bhagyashali Metal, Mumbai |
| Dulbecco's Modified Eagles Medium | Hi-Media, Mumbai |
| Foetal Bovine Serum | Gibco, Mumbai |
| Artificial Saliva | - |
| Calcium Hydroxide | Molychem Industries, Mumbai |
| *Porphyromonas Gingivalis* Bacterial Strain | ATCC33277 |
| Brain Heart Infusion broth | Hi-Media, Mumbai |
| Blood agar base | Hi-Media, Mumbai |
| Glycine based Prophylaxis Powder | 3M ESPE Clinpro, USA |
| Water Soluble Chitosan powder | Pelican Biotech Chemical Labs, Kerela |
| Tetracycline hydrochloride | - |
| MG-63 osteoblast like cell lines | NCCS, Pune |
| MTT Reagent | Hi-Media, Mumbai |
| Dimethyl Sulfoxide (DMSO) | - |
| Phosphate Buffered Saline (PBS) | Hi-Media, Mumbai |
| Trypsin enzyme | Hi-Media, Mumbai |
| Tryphan Blue stain | Sisco Research Laboratories, Mumbai |

**TABLE 2: EQUIPMENTS USED IN THE STUDY**

| EQUIPMENT | MANUFACTURER |
|---|---|
| Ultraviolet Chamber | Laminar Air Flow |
| 24-well micro-titer plates | Tarsons, Korea |
| Micropipettes | Riviera Glass Pvt, Ltd., Mumbai |
| Incubation Chamber | Eppendoff |
| Air Powder Abrasive Device | Waldent Air Flow Device |
| Spectrophotometer | Lisa Plus |
| Haemocytometer | KLEU/BSRC |

**Other armamentarium used in the study**

1. Anaerobic jar
2. Inoculating loop
3. Beaker
4. Autoclave

**METHODS**

## A) Formation of Ca-Precipitated Organic Film Layer on the discs

- Sterile titanium discs are incubated with Dulbecco's Modified Eagles Medium with 20% Foetal Bovine Serum at 37 degree Celsius with 5% carbon dioxide and 95% humidity for 24 hours to create a pellicle and facilitate the biofilm formation. (Fig. 21)

- The discs are then immersed in 1ml of artificial saliva for 24 hours along with anti-biotic solution to prevent contamination. (Fig. 22)

- The discs are then incubated in fresh Dulbecco's Modified Eagles Medium with 20% Foetal Bovine Serum for 72 hours.

- The medium is again refreshed and the discs are incubated for another 72 hours.

- The discs are transferred to new well plates and kept in 2ml of 0.02 M saturated Calcium Hydroxide solutions to create calcium precipitation on the discs. (Fig. 22)

**B) <u>Infecting the biofilm with gram negative Porphyromonas Gingivalis</u>**

- Frozen stocks (-80$^0$C) of *Porphyromonas Gingivalis* will be streaked on blood agar plates using aseptic technique.

- These plates will be incubated anaerobically at 37$^0$C for 24 hours.

- A single colony of *Porphyromonas Gingivalis* will be then cultivated in sterile tube containing 10 ml of sterile Brain Heart Infusion (BHI) Broth.

- 100 microlitre of the inoculated colony is mixed in 1 ml of fresh BHI broth and incubated at 37$^0$C for 1 hour.

- From these 200 microlitres is added to each disc and incubated for 48 hours. (Fig.23)

## C) Evaluation of the presence of pathogenic biofilm

- 0.1% of crystal violet solution will be applied to the colonized discs.
- After incubation for 20 minutes, the discs will be washed 3-4 times with Phosphate Buffered Saline to remove the unbound dye. (Fig. 25)

## D) Air Powder Abrasive Treatment of the titanium discs.

- Air Powder Abrasive Treatment of the biofilm coated titanium disc will be carried out on the test groups only with Waldent Air Flow Device using Glycine and Water Soluble Chitosan powder. (Fig. 24)
- This is done in concentric circles from the center of the disc to the periphery for 60 seconds per disc at a distance of 4mm.

## E) Evaluation of removal of the biofilm.

- The crystal violet stain from all the discs will be washed out with 96% Ethanol and the absorption of the resulting violet solution will be measured using a Spectrophotometer.(Fig. 25)

## F) Application of tetracycline on the titanium discs

- The discs are immersed in 2 ml of 30 mg/ml of tetracycline hydrochloride solution for 2-4 minutes to disinfect the disc completely.

## G) Evaluating the proliferation and adhesion of MG-63 cells.

- Discs will be seeded with MG-63 osteoblast like cells in DMEM at a density of $1*10^6/cm^2$ at $37^0C$ for 30 minutes and later incubated with DMEM supplemented with 20% foetal bovine serum in 5% $CO_2$ in a humidified cell culture incubator at $37^oC$ for 48 hours.

## CELL PROLIFERATION

50 micro litre of $1*10^5$ cells /ml cell suspension

↓

Microtitre plate and volume made to 150 microlitre by adding DMEM media

↓

100 microlitre of dilutions is added to wells for 24 hours in the presence of 5% $CO_2$ and $37^o$ Celsius in a $CO_2$ incubator. (Fig. 26)

↓

20 microlitre of 5mg/ml MTT reagent (Tetrazolium Bromide) is added into the wells

↓

Plate kept for 4 hours in an incubator at room temperature

↓

Removal of supernatant without disturbing Formazan crystals and 200 microlitre of Dimethyl Sulfoxide (DMSO) will be added

↓

Optical density is measured at wavelength 492 by spectrophotometry. (Fig. 19 and 26)

↓

Colorimetric assay measures the reduction of tetrazolium bromide by mitochondrial succinate dehydrogenase enzyme

↓

Reduction of MTT in metabolically active cells measures the proliferation of cells.

# CELL ADHESION

Cells grown approximately 80% confluence prior to cell adhesion assay

↓

MG63 cells will be cultured on various titanium discs in 24 hours multi well plate at a density of $1*10^5$ cells. (Fig. 27)

↓

Culture media is removed and wells are washed 3 times with PBS at 37 degree Celsius to eliminate unattached cells

↓

Adherent cells will be treated with trypsin and stained with tryphan blue and counted using a heamocytometer. (Fig.20)

↓

Cell attachment will be expressed as the percentage of the initial number of cells.

## METHODOLOGY WITH FLOWCHART:

SANDBLASTED AND ACID ETCHED TITANIUM DISCS

STERILIZATION DONE WITH UV IRRADIATION FOR 1 HOUR

FORMATION OF A CALCIUM PRECIPITATED ORGANIC FILM LAYER ON THE DISCS

BIOFILM IS THEN INFECTED WITH GRAM NEGATIVE *PORPHYROMONAS GINGIVALIS*

REMOVAL OF THE PATHOGENIC BIOFILM WITH AIR POWDER ABRASIVE TREATMENT USING GLYCINE AND CHITOSAN POWDERS

APPLICATION OF TETRACYCLINE ON THE TREATED TITANIUM DISCS

MG-63 OSTEOBLAST LIKE CELLS ARE CULTURED ON THE TITANIUM DISCS

CELL PROLIFERATION AND CELL ADHESION WILL BE EVALUATED.

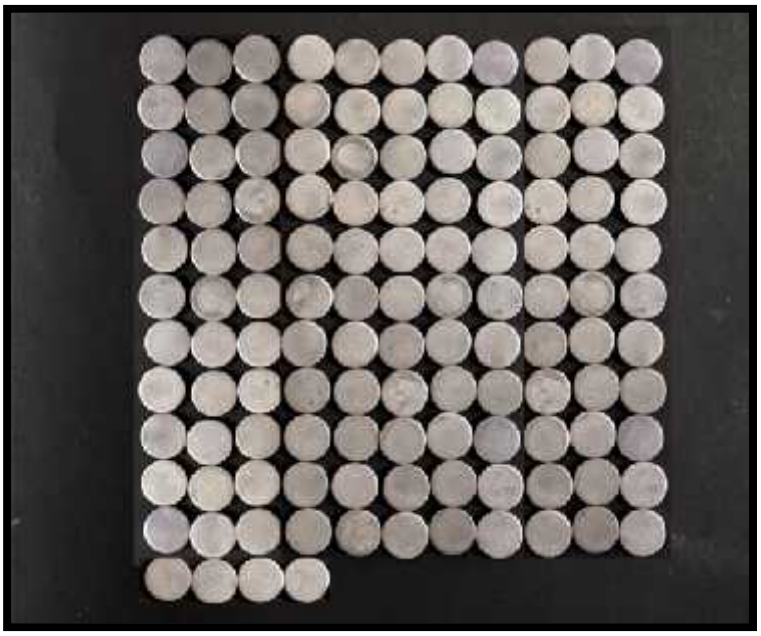


**Fig 1 :- Sand blasted Acid Etched Grade IV Titanium Discs**

**Fig 2 : Dulbecco's Modified Eagle Medium**


**Fig 3 : Foetal Bovine Serum**

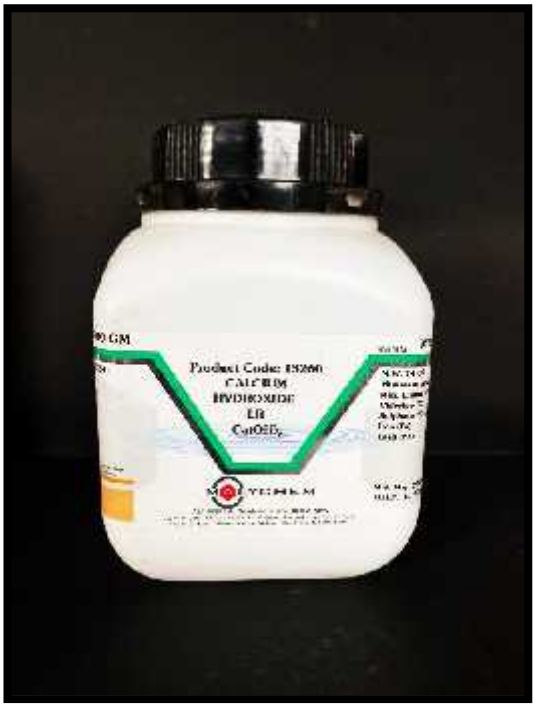


**Fig 4 :- Calcium Hydroxide**


**Fig 5 :-Blood Agar Base (Infusion agar)**

**Fig 6 : Brain Heart Infusion (BHI) Broth**


**Fig 7 : Porphyromonas Gingivalis in BHI broth**


**Fig 8 : Clinpro Glycine Prophy Powder**


**Fig 9 : Water Soluble Chitosan (Pelican Biotech Chemical Lab)**


**Fig 10 : Crystal Violet Stain**

**Fig 11 : Phosphate Buffer Saline**

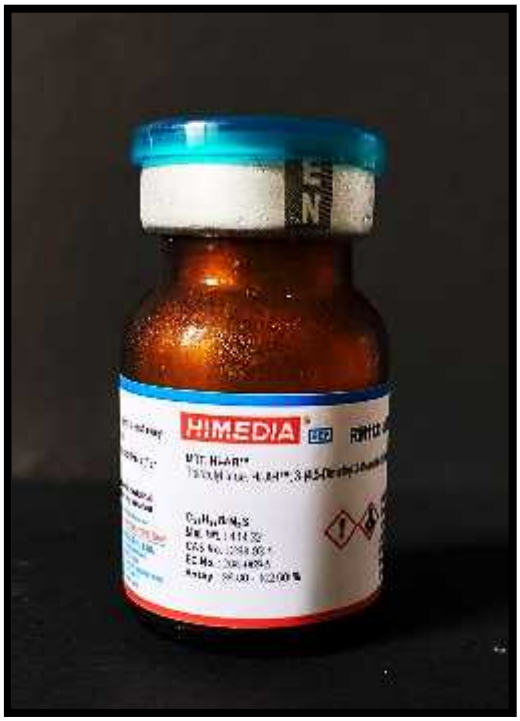



**Fig 12 :- MTT Reagent**

**Fig 13 :- Trypsin**


**Fig 14 :- Tryphan Blue Stain**


**Fig 15 :- Ultraviolet Chamber**


**Fig 16 :- Incubation Chamber**


**Fig 17 :- Bacteriological Incubator**

**Fig 18 :- Anaerobic Jar**


**Fig 19 :- Air Powder Abrasive Device (Waldent)**


**Fig 20 :- Spectrophotometer (LISA PLUS)**

**Fig 21 :- Haemocytometer**

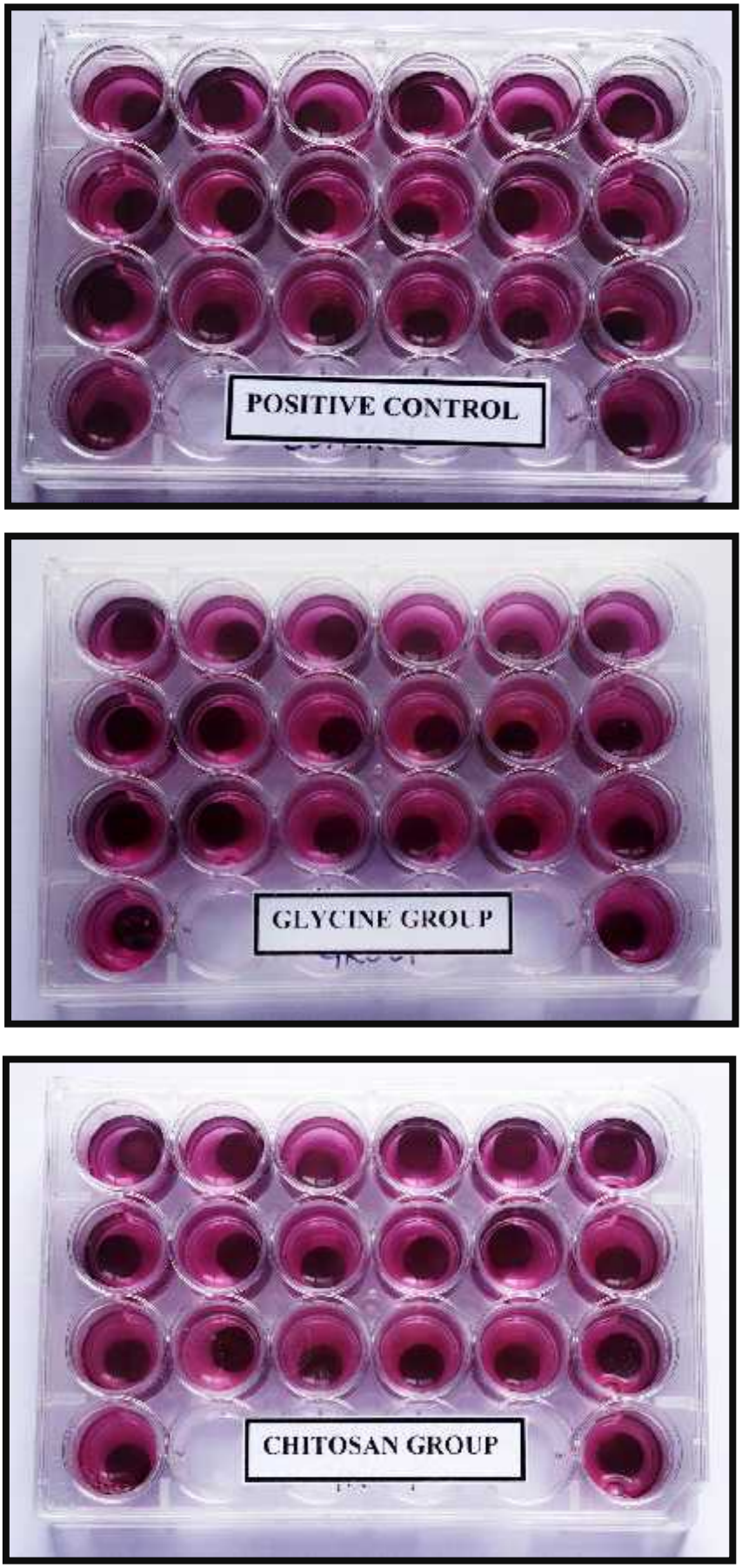



**Fig 22 :- Titanium discs incubated with DMEM and FBS.**

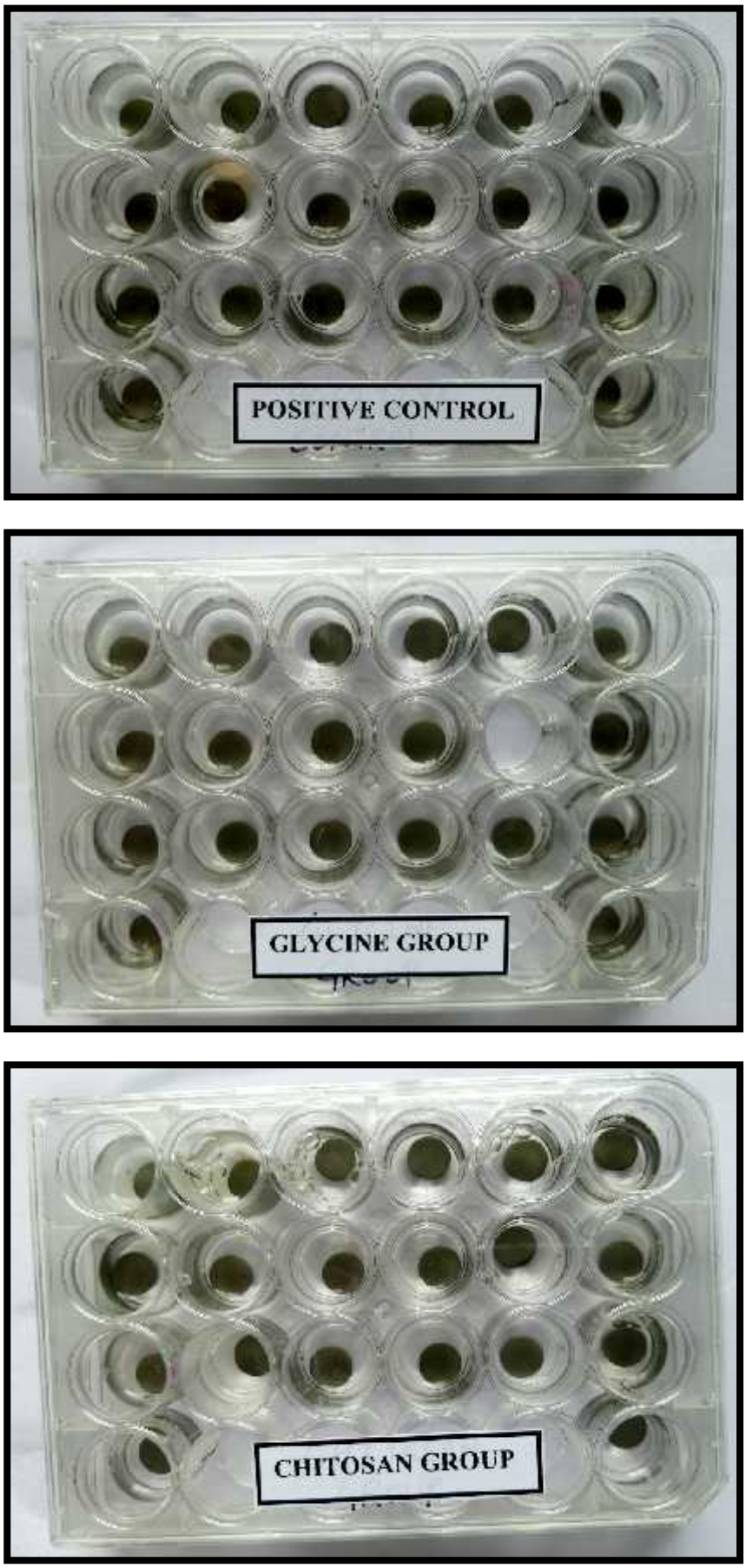



**Fig 23 :- Titanium discs incubated with Artificial saliva and $Ca(OH)_2$ solution**

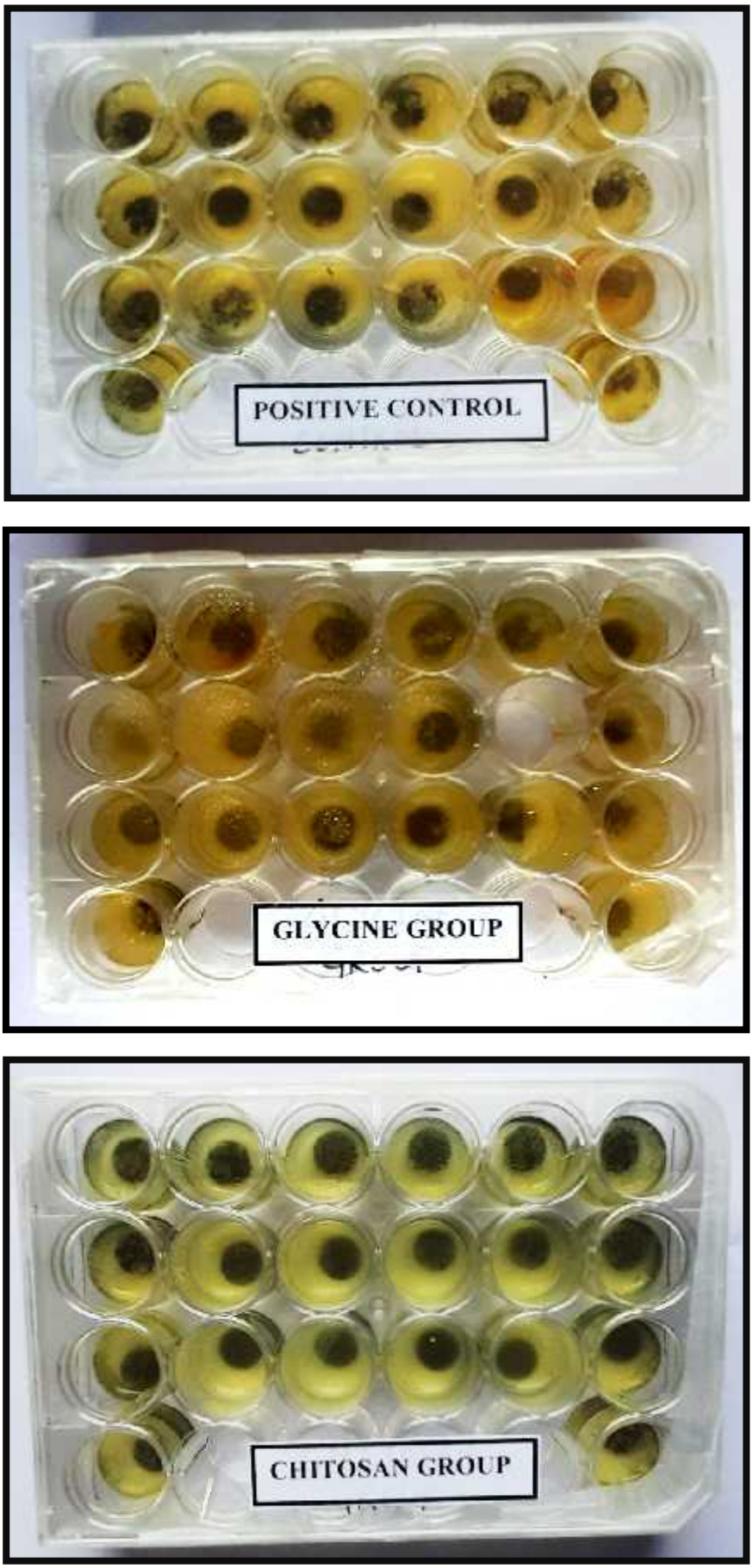



**Fig 24 :- Titanium discs incubated with Porphyromonas gingivalis suspension**

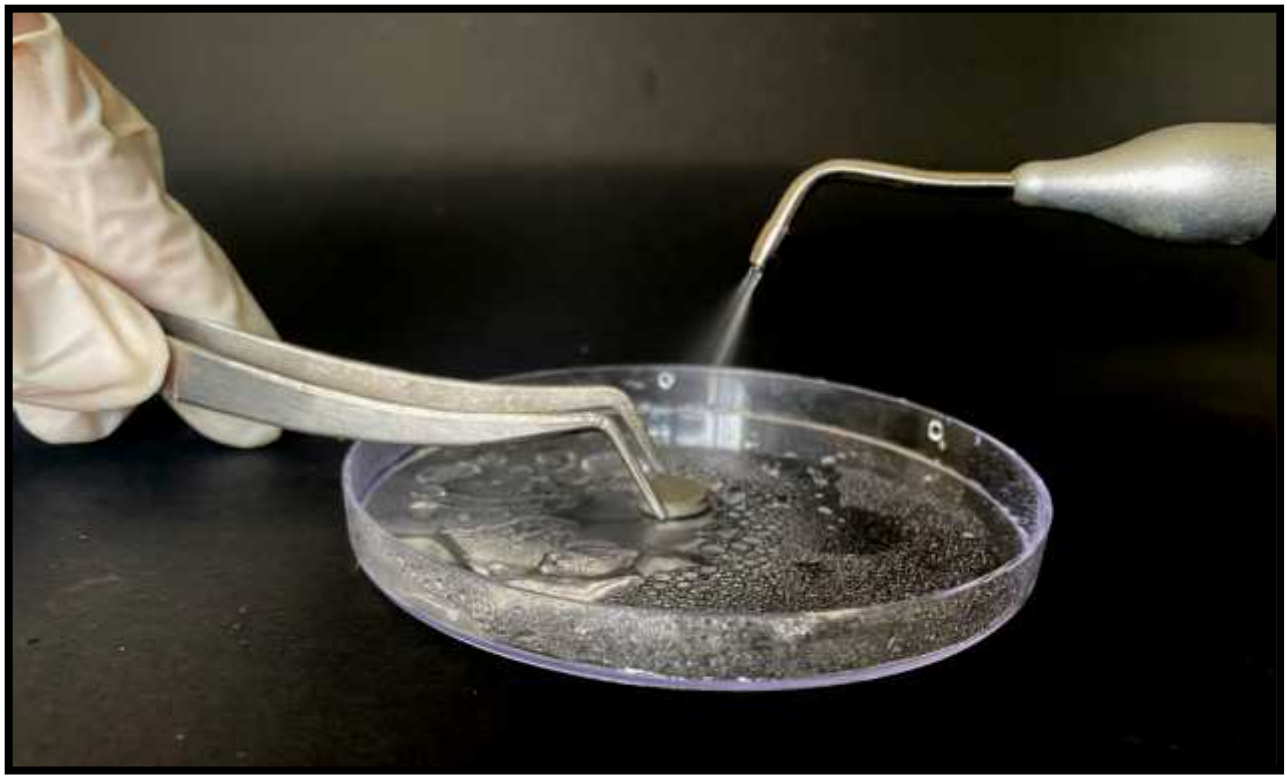


**Fig 25 :- Air Powder Abrasive therapy using Glycine and Water Soluble Chitosan powders**



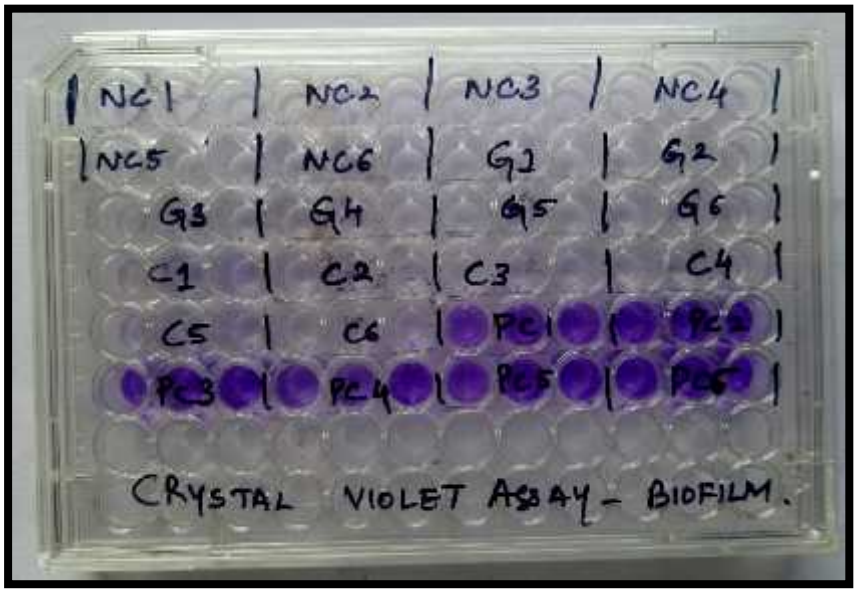
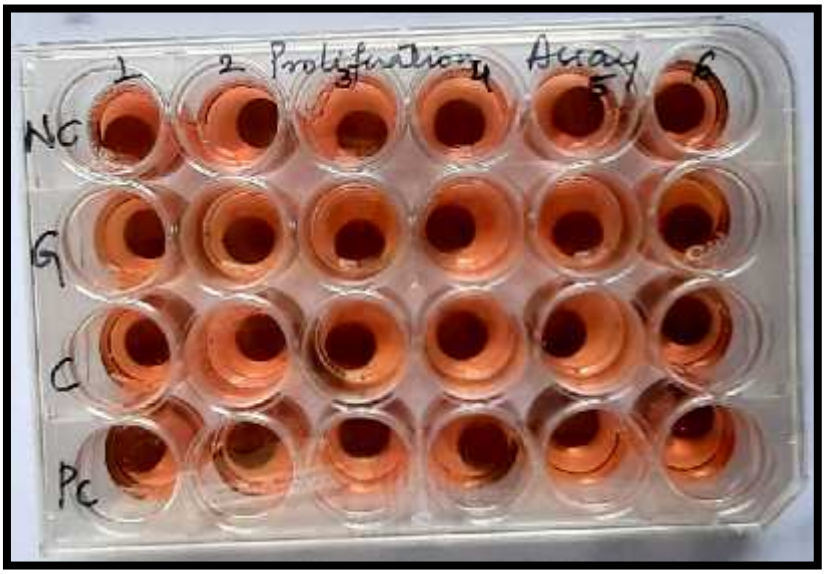



**Fig 26 :- Biofilm Removal evaluated by semi-quantitative analysis using Crystal Violet Stain**



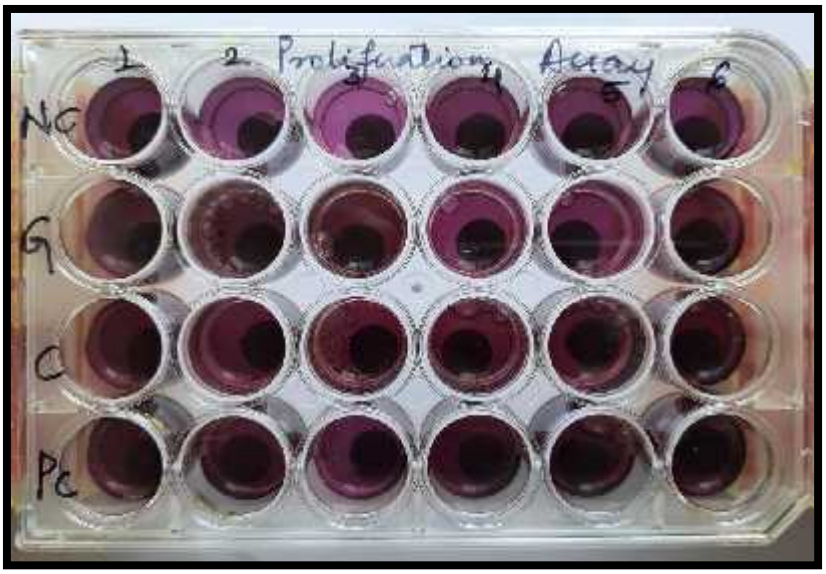




**Fig 27 :- MG – 63 Cell Proliferation evaluated by MTT Assay**

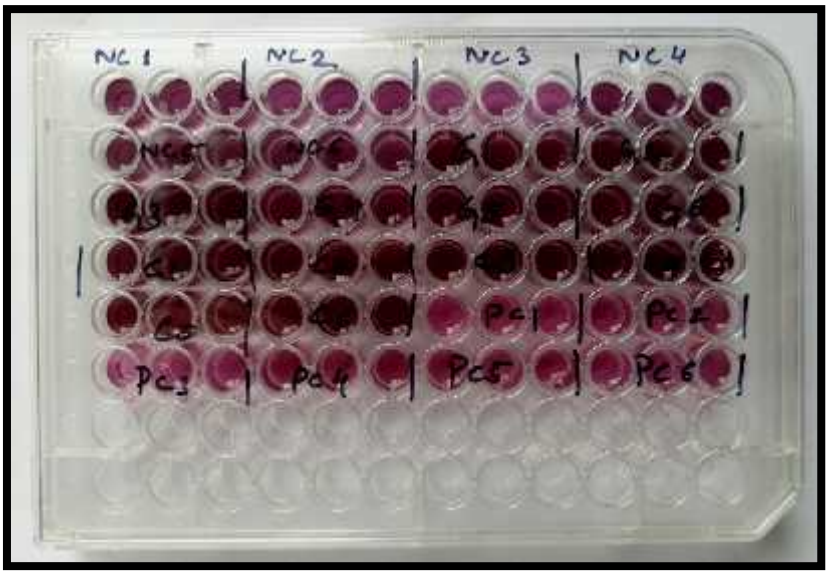



**Fig 28 :- Cell Adhesion evaluated using Haemocytometer**

# RESULTS

The present study evaluates and compares the efficacy of Glycine and Water Soluble Chitosan in removal of pathogenic biofilm from titanium surface using Air Powder Abrasive therapy and subsequent cell proliferation and adhesion of MG-63 osteoblast like cells.

The samples were grouped as follows:

Group PC :- Positive Control

Group NC :- Negative Control

Group G :- APA therapy using Glycine powder

Group C :- APA therapy using Chitosan powder

The removal of pathogenic biofilm was studied by crystal violet assay and was evaluated by calculating the difference between the mean of optical densities of the positive control samples and mean of each test sample and further percentage was calculated compared to the positive control. Cell proliferation or viability was studied by MTT assay as optical densities of each sample and percentage was calculated compared to the optical density of the negative control. Cell adhesion was calculated as the percentage of the initial number of cells.

Statistical Analysis was done by using the following tests:-

- One Way ANOVA -To compare four study groups (PC, NC, G and C) with respect to the percentage of removal of biofilm, cell proliferation and cell adhesion.
- Newman Kewl's Post Hoc - To compare Pair wise comparison of four study groups (PC, NC, G, and C) with respect to the percentage of removal of biofilm, cell proliferation and cell adhesion.

**Table 3: Summary of % of Biofilm Removal in four groups (PC, NC, Glycine and Chitosan)**

| Groups | Min | Max | Mean | SD | SE | 95% CI | |
|---|---|---|---|---|---|---|---|
| | | | | | | Lower Bound | Upper Bound |
| Positive control | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 |
| Negative control | 99.23 | 99.85 | 99.63 | 0.16 | 0.05 | 99.52 | 99.73 |
| Glycine | 71.25 | 85.35 | 81.23 | 4.73 | 1.36 | 78.23 | 84.24 |
| Chitosan | 85.66 | 90.54 | 88.11 | 1.68 | 0.49 | 87.04 | 89.18 |

**Table 4: Comparison of four groups (PC, NC, Glycine and Chitosan) with % of Biofilm Removal by one way ANOVA**

| Sources of variation | Degrees of freedom | Sum of squares | Mean sum of squares | F-value | p-value |
|---|---|---|---|---|---|
| Between groups | 3 | 74415.54 | 24805.18 | 3937.2559 | 0.0001* |
| Within groups | 44 | 277.21 | 6.30 | | |
| Total | 47 | 74692.75 | | | |

*p<0.05

When intergroup comparison was done for Group PC, NC, G and C for the percentage of removal of biofilm using One-way ANOVA, statistically significant results were observed with p-value (<0.05)

**Table 5: Pair wise comparison of four groups (PC, NC, Glycine and Chitosan) with % of Biofilm Removal by Newman Kewl's Post hoc procedures**

| Groups | Positive control | Negative control | Glycine | Chitosan |
|---|---|---|---|---|
| Mean | 0.00 | 99.63 | 81.23 | 88.11 |
| SD | 0.00 | 0.16 | 4.73 | 1.68 |
| Positive control | - | | | |
| Negative control | p=0.0002* | - | | |
| Glycine | p=0.0001* | p=0.0001* | - | |
| Chitosan | p=0.0001* | p=0.0001* | p=0.0001* | - |

*p<0.05

Intergroup comparison by Newman Kewl,s Post hoc procedures was done and statistically significant results were observed in all the groups. Group PC was compared to Group NC, Group G and Group C and statistically significant results were found with p-value as 0.0002, 0.0001 and 0.0001 respectively. When Group NC was compared to Group G and Group C statistically significant results were found with p-value as 0.0001 and 0.0001 respectively. When Group G and Group C were compared the results were statistically significant with p-value as 0.0001. Among all the four groups, the highest mean percentage of biofilm removal was seen with the Group Negative Control as 99.63% which was the sterile group without biofilm formation followed by Group C as 88.11% which was treated with APA therapy using Water Soluble Chitosan, followed by Group G with 81.23% which was treated with APA therapy using Glycine and for Group Positive Control it was 0% as it was not subjected to APA therapy.

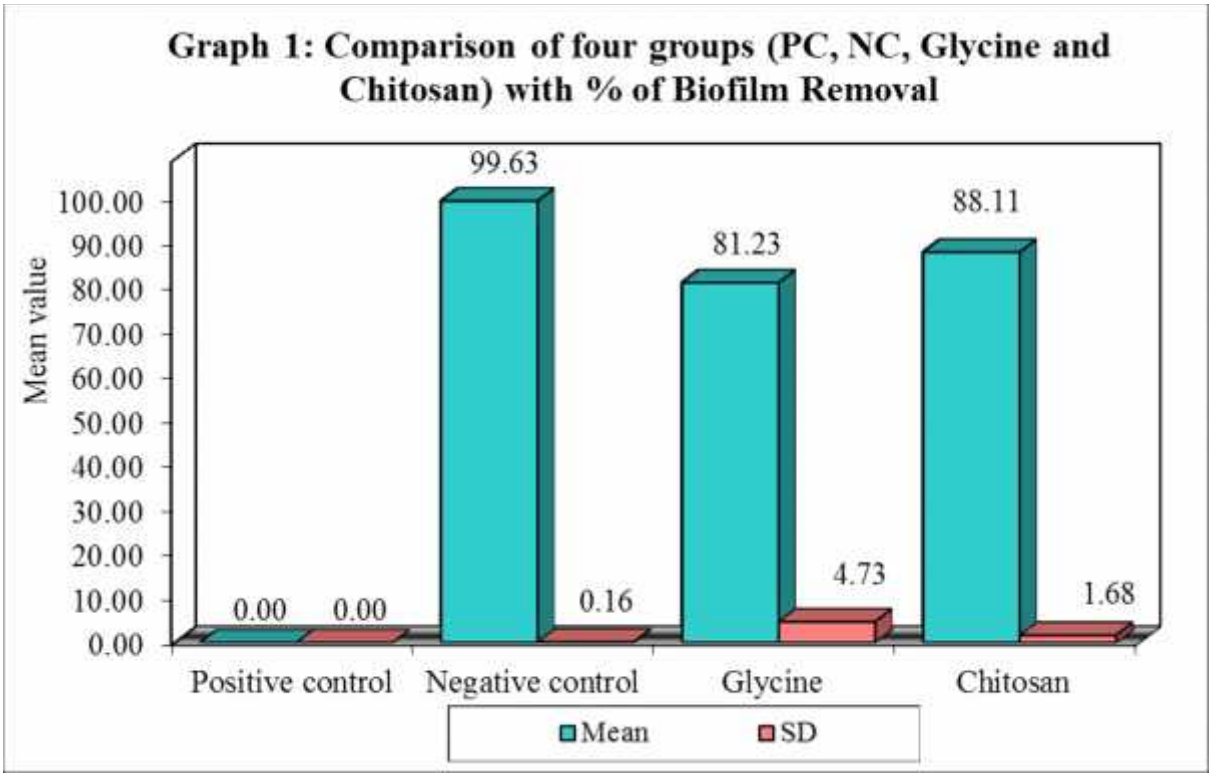

Graph 1: Comparison of four groups (PC, NC, Glycine and Chitosan) with % of Biofilm Removal
Mean value
100.00
90.00
80.00
70.00
60.00
50.00
40.00
30.00
20.00
10.00
0.00
0.00
0.00
99.63
0.16
81.23
4.73
88.11
1.68
Positive control
Negative control
Glycine
Chitosan
Mean
SD

**Table 6: Summary of cell proliferation (% of viability) in four groups (PC, NC, Glycine and Chitosan)**

| Groups | Min | Max | Mean | SD | SE | 95% CI | |
|---|---|---|---|---|---|---|---|
| | | | | | | Lower Bound | Upper Bound |
| Positive control | 33.55 | 48.47 | 40.22 | 4.91 | 1.42 | 37.10 | 43.35 |
| Negative control | 100.00 | 100.00 | 100.00 | 0.00 | 0.00 | 100.00 | 100.00 |
| Glycine | 72.60 | 103.36 | 90.48 | 9.63 | 2.78 | 84.36 | 96.60 |
| Chitosan | 90.29 | 98.77 | 94.08 | 2.02 | 0.58 | 92.79 | 95.36 |

**Table 7: Comparison of four groups (PC, NC, Glycine and Chitosan) with cell proliferation (% of viability) by one way ANOVA**

| Sources of variation | Degrees of freedom | Sum of squares | Mean sum of squares | F-value | p-value |
|---|---|---|---|---|---|
| Between groups | 3 | 27412.85 | 9137.62 | 302.3332 | 0.0001* |
| Within groups | 44 | 1329.84 | 30.22 | | |
| Total | 47 | 28742.69 | | | |

*$p<0.05$

When intergroup comparison was done for Group PC, NC, G and C for the percentage of cell proliferation using One-way ANOVA, statistically significant results were observed with p-value (<0.0001)

**Table 8: Pair wise comparison of four groups (PC, NC, Glycine and Chitosan) with cell proliferation (% of viability) by Newman Kewl's Post hoc procedures**

| Groups | Positive control | Negative control | Glycine | Chitosan |
|---|---|---|---|---|
| Mean | 40.22 | 100.00 | 90.48 | 94.08 |
| SD | 4.91 | 0.00 | 9.63 | 2.02 |
| Positive control | - | | | |
| Negative control | p=0.0002* | - | | |
| Glycine | p=0.0001* | p=0.0004* | - | |
| Chitosan | p=0.0001* | p=0.0116* | p=0.1163 | - |

*p<0.05

Intergroup comparison by Newman Kewl,s Post hoc procedures was done and statistically significant results were observed in all the groups except between Group G and Group C. Group PC was compared to Group NC, Group G and Group C and statistically significant results were found with p-value as 0.0002, 0.0001 and 0.0001 respectively. When Group NC was compared to Group G and Group C statistically significant results were found with p-value as 0.0004 and 0.0116 respectively. However, when Group G and Group C were compared the results were not statistically significant. Among all the four groups, the highest mean percentage of cell proliferation was seen with the Group Negative Control as 100% which was the sterile group without biofilm formation followed by Group C as 94.08% which was treated with APA therapy using Water Soluble Chitosan, followed by Group G with 90.48% which was treated with APA therapy using Glycine and the least percentage of cell proliferation was seen with the Group Positive Control which was 40.22% which was not subjected to APA therapy.

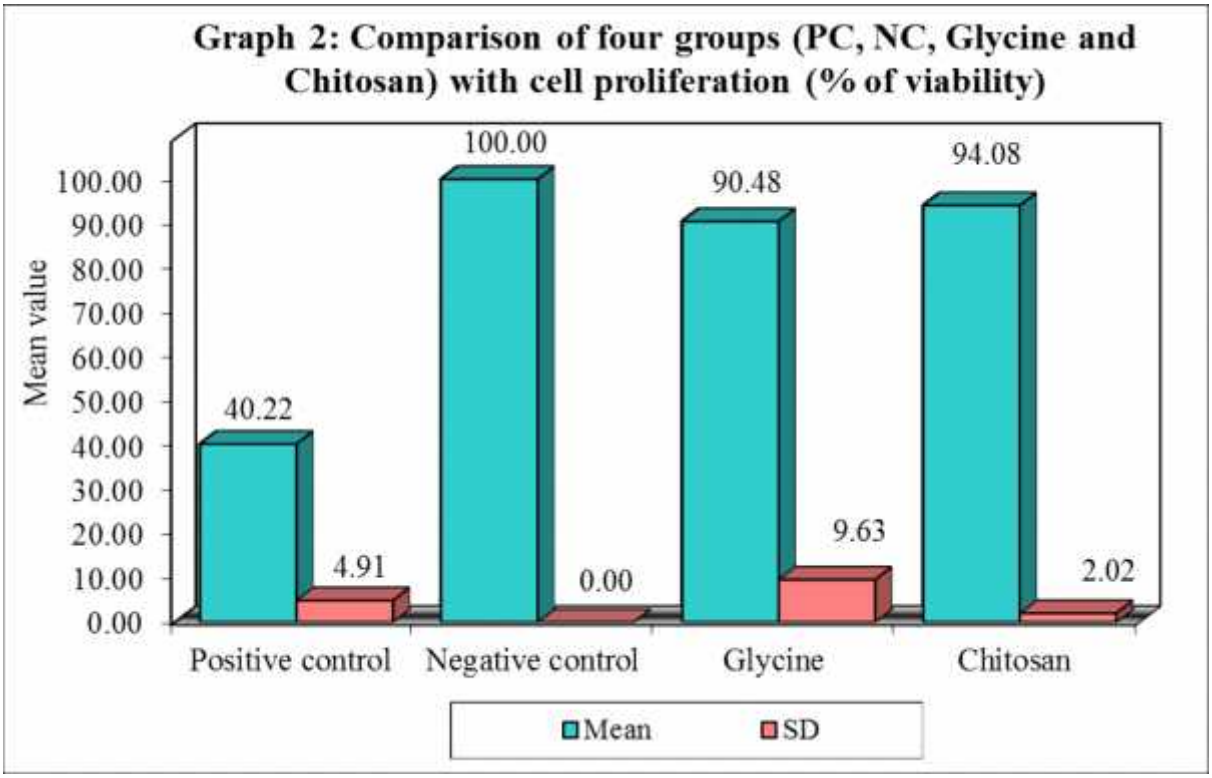

Graph 2: Comparison of four groups (PC, NC, Glycine and Chitosan) with cell proliferation (% of viability)
Mean value
100.00
90.00
80.00
70.00
60.00
50.00
40.00
30.00
20.00
10.00
0.00
40.22
4.91
100.00
0.00
90.48
9.63
94.08
2.02
Positive control
Negative control
Glycine
Chitosan
Mean
SD

**Table 9: Summary of cell adhesion (% cell adhesion) in four groups (PC, NC, Glycine and Chitosan)**

| Groups | Min | Max | Mean | SD | SE | 95% CI | |
|---|---|---|---|---|---|---|---|
| | | | | | | Lower Bound | Upper Bound |
| Positive control | 17.00 | 27.00 | 21.77 | 3.40 | 0.98 | 19.61 | 23.93 |
| Negative control | 40.80 | 68.00 | 53.95 | 9.22 | 2.66 | 48.09 | 59.81 |
| Glycine | 31.50 | 80.00 | 46.22 | 12.03 | 3.47 | 38.58 | 53.86 |
| Chitosan | 40.50 | 57.50 | 49.24 | 4.90 | 1.42 | 46.13 | 52.36 |

**Table 10: Comparison of four groups (PC, NC, Glycine and Chitosan) with cell adhesion (% cell adhesion) by one way ANOVA**

| Sources of variation | Degrees of freedom | Sum of squares | Mean sum of squares | F-value | p-value |
|---|---|---|---|---|---|
| Between groups | 3 | 7438.71 | 2479.57 | 37.4007 | 0.0001* |
| Within groups | 44 | 2917.08 | 66.30 | | |
| Total | 47 | 10355.79 | | | |

*p<0.05

When intergroup comparison was done for Group PC, NC, G and C for the percentage of cell adhesion using One-way ANOVA, statistically significant results were observed with p-value (<0.0001)

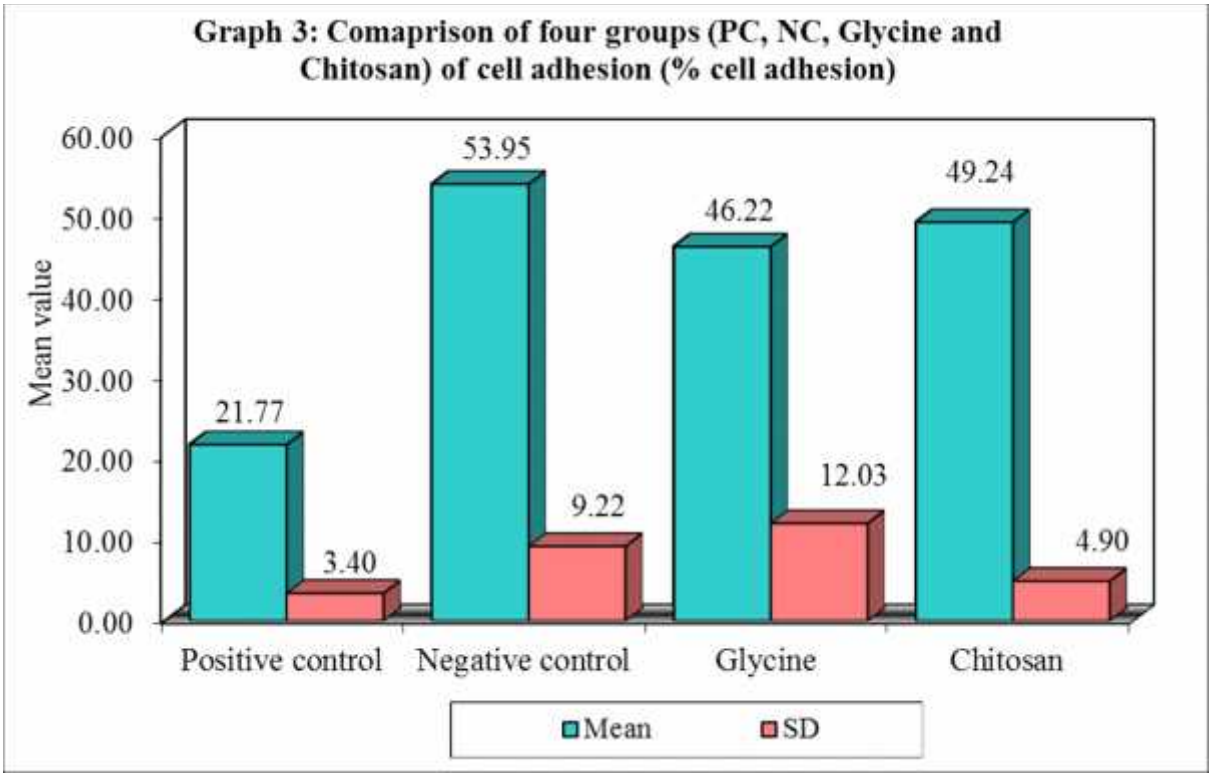
**Table 11: Pair wise comparison of four groups (PC, NC, Glycine and Chitosan) with cell adhesion (% cell adhesion) by Newman Kewl's Post hoc procedures**

| Groups | Positive control | Negative control | Glycine | Chitosan |
|---|---|---|---|---|
| Mean | 21.77 | 53.95 | 46.22 | 49.24 |
| SD | 3.40 | 9.22 | 12.03 | 4.90 |
| Positive control | - | | | |
| Negative control | p=0.0002* | - | | |
| Glycine | p=0.0001* | p=0.0626 | - | |
| Chitosan | p=0.0001* | p=0.1638 | p=0.3679 | - |

*p<p=0.05

Intergroup comparison by Newman Kewl's Post hoc procedures was done and there were no statistically significant differences between the groups except for Group PC. Group PC was compared to Group NC, Group G and Group C and statistically significant results were found with p-value as 0.0002, 0.0001 and 0.0001 respectively. When Group NC was compared to Group G and Group C no statistically significant differences were found. Group G and Group C were compared and the results were not statistically significant. Among all the four groups, the highest mean percentage of cell adhesion was seen with the Group Negative Control as 53.95% which was the sterile group without biofilm formation followed by Group C as 49.24% which was treated with APA therapy using Water Soluble Chitosan, followed by Group G with 46.22% which was treated with APA therapy using Glycine and the least percentage of cell adhesion was seen with the Group Positive Control which was 21.77% which was not subjected to APA therapy.

**Graph 3: Comaprison of four groups (PC, NC, Glycine and Chitosan) of cell adhesion (% cell adhesion)**



# DISCUSSION

Peri-implant lesions or diseases can be classified as either peri-implant mucositis or peri-implantitis, the former being the initial stage. Peri-implant mucositis is basically a reversible inflammation of the soft tissues surrounding a functioning implant whereas peri-implantitis is the further progression which leads to loss of bone around a functioning implant. The prevalence of peri-implant mucositis is higher compared to peri-implantitis at subject as well as at implant level. According to a systematic review by Chun-Teh Lee[51], the prevalence of peri-implant mucositis was 46.83% and 29.84% at subject level and implant level respectively whereas that of peri-implantitis was 19.83% at subject level and 9.25% at implant level. It can be thus inferred that subject based prevalence of peri-implant diseases is higher compared to implant based peri-implant diseases.

Long term success of dental implants replacing missing teeth therefore requires a regular follow up and adequate maintenance is necessary to maintain a healthy peri-implant environment failing which the implants can be rendered non-functional. Peri-implantitis is of greater concern than peri-implant mucositis as it is the further sequel and consists of greater inflammatory response causing tissue injury. Various treatment modalities are being used for debridement of the inflammatory environment around the affected implants ranging from chemical, mechanical or a combination of both the methods which can be carried out non-surgically or surgically. This study is based on the use of Air Powder Abrasive therapy for decontamination of bacterial biofilms on titanium surfaces and further cellular response of MG-63 osteoblast like cells. Apart from the conventional powders which

are generally used for APA therapy, we used water soluble chitosan which is a versatile biopolymer in the form of a fine powder.

The main culprit in the initiation of peri-implant diseases is the colonization of bacterial colonies around the surface of the implant. Microorganisms exist either in free floating state i.e. planktonic bacteria or in the form of biofilm i.e. sessile bacteria. Biofilm represents an organized structure in which microorganisms interact metabolically as a community. Most of the bacteria prefer the biofilm mode of growth as it helps provide nutrient, protect the bacterial community from other competing microorganisms and prevents the spread of antimicrobial agents. The formation of bacterial biofilm around implant surfaces is similar to that of the teeth. Initially a salivary pellicle is formed on the surface within 30 minutes of the implant coming in contact with the oral environment followed by bacterial adhesion of initial colonizers which is later followed by cell to cell attachment of secondary colonize rs.[52] Biofilms have been related to about 65% of the periodontal and peri-implant diseases.[53]

The main objective of the decontamination methods is complete eradication or reduction of the microbial load around the implant surfaces and thus elimination of the biofilms harbouring them. This re-establishes the biocompatibility of the titanium surfaces on which the host cells can adhere and proliferate thereby restoring healthy peri-implant conditions. When peri-implantitis sets in, there is a change in the number and type of bacteria around the implant surface. The gram positive bacteria which are generally seen in a healthy condition are replaced by gram negative anaerobic bacteria which consist of the red complex like P. gingivalis, Treponema denticola, T. forsythia etc. and orange complex like P. intermedia and Fusobacterium nucleatum, along with Aggregatibacter actinomycetemcomitans.[54]

In our study we created calcium precipitated organic biofilm layer on which P.Gingivalis bacterial strain (ATCC33277) was cultured. According to Yoshinari[54], electrical charge on the titanium surfaces influences the bacterial adhesion. Calcium ions ($Ca^{+2)}$ from the calcium hydroxide solution get precipitated on the negatively charged titanium surface which in turn attracts the negatively charged bacteria thereby facilitating in formation of an adherent biofilm. P. Gingivalis is considered as a prime bacteria in the pathogenesis of periodontal and peri-implant disease. It has been found in 85% of the subgingival plaque of periodontitis and peri-implant diseases. The strain ATCC33277 used in the study consists of abundant fimbriae's that has the ability to attach adherently to the host cells or to the implant surfaces whether smooth or rough.[55]

Currently, many decontaminating methods are available for treatment of peri-implant diseases. However, no specific decontamination modality has proven to be superior or more efficient in re-establishing the biocompatibility of the contaminated titanium surface according to Claffey et al [56]. In this study we have utilized Air Powder Abrasive therapy, also known as Air Polishing for elimination of the bacterial contaminants from the surface of the titanium discs. Efficient debridement of bacterial biofilms and endotoxins has been demonstrated in various in-vivo and in-vitro studies using air polishing. Dennison[17] reported that APA therapy has been more effective in removal of radioactive endotoxin obtained from P.Gingivalis from titanium surfaces that were hydroxyapatite coated, plasma spayed or machined when compared to application of citric acid by cotton pellet.

Sahrmann evaluated the efficacy of APA therapy in removal of permanent non covering ink from surfaces of implants kept in artificial defect models. Residual ink

stains were found on implant surfaces of all four models types which explain the partial inaccessibility of the powders to the implants surfaces especially in narrow defects. The area under the threads of the implant surfaces was considered as a problematic area to be cleaned. However it was possible to access up to 90% of the exposed implant surface with the APA therapy using glycine powder.[23] Compared to gracey curettes and ultrasonic scalers, APA therapy using glycine proved to be a more efficient and viable option for areas that are inaccessible to curettes or ultrasonic scalers.[21,30,32,44.]

APA therapy involves mechanical debridement due to the direct impact of the pressurized stream of powder along with air and water. Along with mechanical debridement, the type of powder used also influences the further cellular response of the host tissue and may prevent further re-colonization of the pathogenic microorganisms. It has been demonstrated that APA therapy using Erythritol powder decreased the attachment of Streptococcus strains and also reduce the growth of P.Gingivalis and S.Gordonii by inhibiting different metabolic pathways.[40] One of the advantages of using APA therapy is that along with the central beam of the air polishing device, a radial corona of secondary jets arises parallel to the surface to be treated. These secondary jets or beams are caused due to ricochet effect that has an important influence on the instrument's efficacy. This effect can prove to be beneficial in decontaminating procedures especially in areas that are difficult to access.[57]

As mentioned earlier, the type of powder plays a major role in the successful execution of the APA treatment. Factors regarding the powder such as size, abrasiveness, anti-microbial activity, solubility and biocompatibility are to be

considered when using APA therapy for treating peri-implantitis. In our study we have utilized the conventionally available amino acid glycine and water soluble chitosan to be used along with the air polishing device. Glycine is generally preferred as the particle size used for air polishing is around 25µ, has less abrasiveness compared to sodium bicarbonate and is soluble in oral fluids. Drago et al[25] in his in-vitro study has demonstrated bacteriostatic activity of glycine, reducing the percentage of surviving cells by 30% for C.Albicans and B.Fragilis and 15% for S.Aureus. Matsubura et al[46] has shown that use of sodium bicarbonate causes increase in the surface roughness of the implant which facilitate further biofilm formation and it is also associated with causing damage to the gingival tissues according to Magda et al.[40]

Chitosan [(1----4) 2-amino-2-deoxy-&-Glucan] is a unique polysaccharide obtained from chitin. Chitin is one of the most abundant aminopolysaccharide found in nature. Functionally it is related to cellulose and collagen and thus they form the bases of the principal skeletal systems of animals which are collagenous and the chitinous.[58] Chitosan has been used as an antimicrobial against a wide range of microorganisms like bacteria, fungi, algae yeast etc. in numerous in-vivo and in-vitro studies. It has proven antimicrobial activity against pathogenic bacteria's associated with periodontitis and those associate with peri-implant diseases like P.Gingivalis, S.Mutans and A.Actinomycetemcomitans. These are predominantly associated with the biofilm formation and therefore utilization of chitosan as a powder in the APA therapy can prove to be beneficial in future. Some other properties that are beneficial are biodegradability, biocompatibility, bioactivity, anti-inflammatory action and ability to accelerate wound healing.[59]

## A) BIOFILM REMOVAL

In order to evaluate biofilm removal SLA grade IV titanium discs were subjected to formation of calcium precipitated bacterial biofilm using Porphromonas gingivalis. Discs in the test group (Group G and Group C) were subjected to air polishing using glycine and water soluble chitosan. The efficacy of both the powders in elimination of the biofilm was evaluated by semi-quantitative analysis using crystal violet stain and spectrophotometer. The absorbance of residual crystal violet stain depicted the amount of biofilm removal that has been undergone in all the four groups. Results showed that both glycine and water soluble chitosan were effective in removal of biofilm by 81.23% and 88.11% respectively. The percentage of biofilm removal on the positive control (Group PC) was considered to be 0% as it was not subjected to air polishing and the difference between the optical densities of the test and control groups was found to be significant. Negative control (Group NC) failed to get stained by crystal violet due to the absence of biofilm; however, some residual stain got incorporated on the rough surfaces of the SLA titanium discs. Due to this, the result for percentage of biofilm removal for the Group NC was 99.63%.

Chitosan proved to be better at biofilm removal upto a certain extent compared to glycine but the difference was not significantly large. According to our assumption water soluble chitosan proved to be more efficient compared to glycine due to the larger sized particles of chitosan which was in the range of 70µ-80µ compared to the much smaller sized glycine particles which was 25µ. This is in accordance with the results obtained by a study conducted by Matsubura et al [46,] in which air polishing was carried out using sodium bicarbonate (40µ-65µ), glycine (25µ) and erythritol (14µ) to remove permanent ink from surfaces of implants. It was found that residual ink found

on implants treated using sodium bicarbonate was significantly lower compared to glycine and erythritol, however, leading to increase in the surface roughness and change in the surface topography. The use of chitosan as a decontaminating material in treatment of peri-implantitis is scarce. In a case series conducted by Wohlfahrt et al [39], reduction in peri-implant inflammation was observed after 2,4,12 and 24 weeks after using a chitosan brush in a dental drill piece. Complete elimination of inflammation was difficult to achieve as the chitosan brush was used non-surgically, however, reduction in the parameters of peri-implant inflammation was evident after 6 months from baseline.

In another in-vitro study by Larsen et al [36], titanium curettes, Er:YAG laser and chitosan brush were used for decontamination of implant surfaces contaminated with P.gingivalis. It was found that all three decontamination methods were equally efficient in reducing the bacterial counts from micro-textured SLA implant surfaces. Titanium curettes significantly altered the surface topography compared to chitosan brush and Er:YAG laser.

However, in a randomized control trial conducted by Koldsland et al [48], unfavourable results were observed when titanium curettes and chitosan brush were used for maintenance after surgical treatment of peri-implantitis. The bleeding on probing and inflammation was high 6 months after the surgical treatment and maintenance program for peri-implantitis. Both titanium curette and chitosan brush failed to achieve complete eradication of inflammation.

In the present in-vitro study, however, positive results were obtained concerning the removal of biofilm from SLA titanium surfaces when water soluble chitosan was used. Given the heterogeneity and lack of research regarding potency of

chitosan as a powder that can be used for biofilm removal, it is necessary that further studies and research should be performed that can enable us to use this versatile biopolymer in treatment of peri-implantitis and advance the implant dentistry.

## B) CELL ADHESION AND PROLIFERATION

Complete eradication of the biofilm, cytotoxic products and other deposits that have been developed on the implant surfaces is the basic prerequisite for the host cells to attach and proliferate on the implant surface. The original implant condition has to be restored so as to make the implant surface biocompatible and to make it viable for bone regeneration to take place. APA therapy is chosen as the mode of decontamination due to its advantages like efficient biofilm removal, absence of any change in surface topography and not causing damage to the surrounding tissues and potency to re-establish biocompatibility when used with the appropriate powders.

Rosen [42] demonstrated a decontamination protocol which involved the usage of ultrasonic scalers followed by APA therapy using glycine and later burnishing the implant surface using citric acid and cotton pellet. The protocol favoured in cell attachment of human osteoprogenitor cells on the decontaminated implant surfaces whereas the implants that didn't receive any treatment lacked cell attachment due to the presence of biofilm, microorganisms, and calculus. Presence of smear layer on the implant surface prevents the host cells from attaching to the solid implant surface according to the theory of contact guidance of cells.

In the present study, cell attachment was evaluated using haemocytometer by counting the number of cells manually [60] and reported as number of cells x 2 x $10^4$. The untreated titanium discs (Group PC) revealed much lower values of MG-63 adhesion compared to other three groups. The % of cell adhesion for the Group PC

was 21.77% compared to 53.95%, 49.24% and 46.22% for Group NC, G and C respectively. Cell proliferation was evaluated using MTT assay which is a colorimetric assay that records the change in the colour of the MTT reagent using spectrophotometer. The percentage of cell proliferation for the negative control (Group NC) was considered to be 100% according to the formula used and that of the Groups PC, C, and G were 40.22%, 94.08% and 90.48%, respectively. The above results prove that the APA therapy has been successful in restoring the biocompatibility of the contaminated titanium discs as the adhesion and proliferation in the positive controls was significantly lower.

According to Ceyline [22], employing APA therapy for treatment of peri-implantitis can lead to deposition of the powders on the titanium surfaces which can interfere with the re-osseointegration process if the powders are not biocompatible. Remnants of biocompatible and osteoconductive powders like hydroxylapatite and tricalcium phosphate were found on the titanium surfaces which was confirmed by SEM and EDS analysis which may aid in the re-osseointegration process and increase the bone-implant contact.

Therefore in our study it can be speculated that the presence of chitosan on the titanium surface might be the reason for the higher proliferation and cell adhesion compared to the glycine group. Chitosan sponge along with platelet derived growth factor-BB displayed bone healing efficacy, regeneration and showed sufficient cellular adaptability which was speculated to be used as a future alternative in periodontal regenerative therapy.[61] has the ability to activate osteoblasts and increase osteogenesis. Chitosan 3D-scaffolds in the form of sponges and hydrogels are also prepared for the purpose of tissue engineering and wound healing respectively. At the

same time chitosan is a biodegradable material due to the presence of glycosidic bonds which can be broken by several proteases and lysozymes [62] which enables the material to be used as a bone graft material. Francesco [63] revealed decrease in the growth of bacterial cultures and stimulated osteoblast to produce higher amount of bone extracellular matrix along with biomineralization when titanium surfaces were coated with chitosan/PEO/bioactive glass nanofiber composites. Along with the anti-bacterial properties of chitosan, the titanium discs were immersed in tetracycline solution (30mg/ml) which may have reinforced the anti-bacterial activity leading to favourable cellular activity in both the test groups.[65, 66]

The overall findings of the present study indicate that Air Powder Abrasive therapy when used with Glycine and Water Soluble Chitosan can effectively remove biofilms and detoxify the titanium surfaces, thereby restoring the biocompatibility and inducing re-osseointegration. Within the limitations of the present research there is a scope for improvement and further research can be done to evaluate and compare different methods for the treatment of peri-implantitis that can help us to provide an elevated implant treatment for our patients.

# SCOPE OF THE STUDY

1. A study can be performed in which the change in the surface topography of titanium implants can be evaluated with APA using Water Soluble Chitosan powders of different particles sizes.
2. A study can be performed to decontaminate titanium surfaces using different methods like lasers, curettes, chemical agents and compare it with APA therapy using the water soluble chitosan.
3. An in-vivo animal study can be performed to evaluate the host tissue response of water soluble chitosan as a material used for APA therapy.
4. MIC and MBC of water soluble chitosan can be evaluated.

# LIMITATIONS OF THE STUDY

1. The present study is an in-vitro study and therefore host tissue response towards water soluble chitosan was not evaluated.
2. The biofilm was prepared by using Porphyromonas Gingivalis which is a secondary colonizer and has less adherence towards any surface compared to the initial colonizer like S.Mutans etc. Therefore, removal of a loosely attached biofilm could be easier compared to a more adherent biofilm consisting of initial colonizers.
3. The effect of the APA therapy on the titanium surface roughness was not evaluated.

# CLINICAL IMPLICATIONS

1. The present study has showed that water soluble chitosan was equally efficient in removal of biofilms when compared to glycine and therefore this material can be used to decontaminate implants with peri-implant lesions.
2. Water soluble chitosan was able to restore the biocompatibility of contaminated titanium surfaces and was favourable for the proliferation and adhesion of MG-63 osteoblast like cells thus indicating that it can be used for the treatment of peri-implantitis in future.

# CONCLUSIONS

Within the limitations of this in-vitro study and the results acquired, following conclusions could be drawn:-

1. APA therapy using Water Soluble Chitosan was effective in sufficient biofilm removal from contaminated titanium surfaces.
2. APA therapy using Water Soluble Chitosan was effective in restoring the biocompatibility of titanium surfaces and was favourable for re-osseointeration.

# SUMMARY

The present study was conducted to evaluate and compare the ability of APA therapy using water soluble chitosan and glycine to remove bacterial biofilms from titanium surfaces and re-establish its biocompatibility.

Sand blasted acid etched grade IV titanium discs were used to carry out the experiments. 144 titanium discs were divided into 4 groups which were categorized as positive control (Group PC), negative control (Group NC), glycine treated (Group G) and water soluble chitosan treated (Group C). They were subjected to biofilm formation except for Group NC and further biofilm removal was carried out by Air Powder Abrasive therapy using glycine and water soluble chitosan powders which was evaluated using spectrophotometer. Later, cell adhesion and cell proliferation of MG-63 osteoblast like cells was studied using haemocytometer and MTT Assay respectively.

The null hypothesis stated that there is no difference in biofilm removal and cellular response of MG-63 cells when treated with APA therapy using water soluble chitosan and glycine.

The titanium discs were coated subjected to crystal violet staining after biofilm formation and optical density of the residual crystal violet stain was evaluated using a spectrophotometer to study the effect of APA therapy on biofilm removal. Cell adhesion was done manually by counting the number of cells attached on the titanium discs using a haemocytometer. Cell proliferation was studied using MTT Assay and the change in the colour of the MTT reagent was studied under a spectrophotometer.

The data obtained was subjected to statistical analysis using SPSS software version 20. The statistical analysis performed by One Way ANOVA and Newman Kewl's Post Hoc test for comparison of biofilm removal, cell adhesion and proliferation in the four groups.

Within the limitations of the study, it was observed that APA therapy using water soluble chitosan was effective in removal of biofilm and further restore the biocompatibility thereby favouring the cell adhesion and cell proliferation of MG-63 osteoblast like cells.

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# ANNEXURE - I - ETHICAL CLEARANCE CERTIFICATE

**Research and Ethics Committee**
**KLE V K INSTITUTE OF DENTAL SCIENCES**
**KLE University**
Accredited 'A' Grade by NAAC Placed in Category 'A' by MHRD (GoI)
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Sl. No.: 1207

**CERTIFICATE**

*This is to Certify that the synopsis titled*

To evaluate and compare the efficacy of glycine and chitosan powder to regain the biocompatibility of contaminated titanium surfaces: an in-vitro study *Submitted by*

*Dr.* _____ *P. G. Student / Staff, Guided by* _____ *from Department of* Prosthodontics and Crown & Bridge *has been critically evaluated by committee members and granted ethical clearance to conduct the above mentioned study*

**Date :** 24/06/2019

**Member Secretary**
Research and Ethical Committee
KLEVK Institute of Dental Sciences
Belagavi

**Chairman**
Research and Ethical Committee
KLEVK Institute of Dental Sciences
Belagavi

# ANNEXURE - II

## Mean percentages of biofilm removal

| Groups | Samples | OD | Mean | % Biofilm Removal |
|---|---|---|---|---|
| PC | 1 | 1.374 | 1.29725 | 0 |
| | 2 | 1.223 | | |
| | 3 | 1.235 | | |
| | 4 | 1.355 | | |
| | 5 | 1.357 | | |
| | 6 | 1.22 | | |
| | 7 | 1.244 | | |
| | 8 | 1.361 | | |
| | 9 | 1.351 | | |
| | 10 | 1.231 | | |
| | 11 | 1.368 | | |
| | 12 | 1.248 | | |

# ANNEXURE - II

## Mean percentages of biofilm removal

| Groups | Samples | OD | Mean | % Biofilm Removal |
|---|---|---|---|---|
| NC | 1 | 0.001 | 0.01 | 99.229139 |
| | | 0.012 | | |
| | | 0.017 | | |
| | 2 | 0.009 | 0.006 | 99.537483 |
| | | 0.003 | | |
| | | 0.006 | | |
| | 3 | 0.001 | 0.00433 | 99.66596 |
| | | 0.006 | | |
| | | 0.006 | | |
| | 4 | 0.003 | 0.00467 | 99.640265 |
| | | 0.007 | | |
| | | 0.004 | | |
| | 5 | 0.005 | 0.00367 | 99.717351 |
| | | 0.004 | | |
| | | 0.002 | | |
| | 6 | 0.007 | 0.00567 | 99.563179 |
| | | 0.004 | | |
| | | 0.006 | | |
| | 7 | 0.003 | 0.002 | 99.845828 |
| | | 0.001 | | |
| | | 0.002 | | |
| | 8 | 0.009 | 0.006 | 99.537483 |
| | | 0.006 | | |
| | | 0.003 | | |
| | 9 | 0.001 | 0.00467 | 99.640265 |
| | | 0.008 | | |
| | | 0.005 | | |
| | 10 | 0.003 | 0.002 | 99.845828 |
| | | 0.002 | | |
| | | 0.001 | | |
| | 11 | 0.009 | 0.00433 | 99.66596 |
| | | 0.001 | | |
| | | 0.003 | | |
| | 12 | 0.008 | 0.005 | 99.614569 |
| | | 0.005 | | |
| | | 0.002 | | |

# ANNEXURE - II

## Mean percentages of biofilm removal

| Groups | Samples | OD | Mean | % Biofilm Removal |
|---|---|---|---|---|
| G | 1 | 0.259 | 0.218 | 83.195221 |
| | | 0.204 | | |
| | | 0.191 | | |
| | 2 | 0.188 | 0.21067 | 83.760519 |
| | | 0.169 | | |
| | | 0.275 | | |
| | 3 | 0.208 | 0.21 | 83.81191 |
| | | 0.199 | | |
| | | 0.223 | | |
| | 4 | 0.251 | 0.33867 | 73.893493 |
| | | 0.36 | | |
| | | 0.405 | | |
| | 5 | 0.364 | 0.278 | 78.570052 |
| | | 0.252 | | |
| | | 0.218 | | |
| | 6 | 0.209 | 0.19167 | 85.225156 |
| | | 0.19 | | |
| | | 0.176 | | |
| | 7 | 0.198 | 0.21633 | 85.353633 |
| | | 0.212 | | |
| | | 0.239 | | |
| | 8 | 0.261 | 0.373 | 71.246868 |
| | | 0.441 | | |
| | | 0.417 | | |
| | 9 | 0.334 | 0.28433 | 78.08184 |
| | | 0.294 | | |
| | | 0.225 | | |
| | 10 | 0.183 | 0.19 | 85.353633 |
| | | 0.191 | | |
| | | 0.196 | | |
| | 11 | 0.277 | 0.22733 | 82.47575 |
| | | 0.194 | | |
| | | 0.211 | | |
| | 12 | 0.294 | 0.20967 | 83.837605 |
| | | 0.153 | | |
| | | 0.182 | | |

# ANNEXURE - II

## Mean percentages of biofilm removal

| Groups | Samples | OD | Mean | % Biofilm Removal |
|---|---|---|---|---|
| C | | 0.109 | | |
| | | 0.152 | | |
| | 1 | 0.157 | 0.13933 | 89.259331 |
| | | 0.179 | | |
| | | 0.263 | | |
| | 2 | 0.116 | 0.186 | 85.661977 |
| | | 0.151 | | |
| | | 0.176 | | |
| | 3 | 0.146 | 0.15767 | 87.846085 |
| | | 0.139 | | |
| | | 0.117 | | |
| | 4 | 0.114 | 0.12333 | 90.492709 |
| | | 0.175 | | |
| | | 0.174 | | |
| | 5 | 0.19 | 0.17967 | 86.15019 |
| | | 0.187 | | |
| | | 0.161 | | |
| | 6 | 0.166 | 0.17133 | 86.792574 |
| | | 0.123 | | |
| | | 0.141 | | |
| | 7 | 0.152 | 0.13867 | 89.310721 |
| | | 0.119 | | |
| | | 0.126 | | |
| | 8 | 0.123 | 0.12267 | 90.5441 |
| | | 0.181 | | |
| | | 0.168 | | |
| | 9 | 0.125 | 0.158 | 87.820389 |
| | | 0.193 | | |
| | | 0.152 | | |
| | 10 | 0.161 | 0.16867 | 86.998137 |
| | | 0.219 | | |
| | | 0.141 | | |
| | 11 | 0.153 | 0.171 | 86.818269 |
| | | 0.188 | | |
| | | 0.105 | | |
| | 12 | 0.112 | 0.135 | 89.593371 |

# ANNEXURE - III

## Mean percentages of cell adhesion

| Groups | Samples | Cells/disc | % cell adhesion |
|---|---|---|---|
| PC | 1 | 205000 | 20.5 |
| | 2 | 215000 | 21.5 |
| | 3 | 270000 | 27 |
| | 4 | 175000 | 17.5 |
| | 5 | 235000 | 23.5 |
| | 6 | 170000 | 17 |
| | 7 | 240000 | 24 |
| | 8 | 240000 | 24 |
| | 9 | 183000 | 18.3 |
| | 10 | 182000 | 18.2 |
| | 11 | 254000 | 25.4 |
| | 12 | 243000 | 24.3 |
| NC | 1 | 415000 | 41.5 |
| | 2 | 680000 | 68 |
| | 3 | 595000 | 59.5 |
| | 4 | 508000 | 50.8 |
| | 5 | 649000 | 64.9 |
| | 6 | 567000 | 56.7 |
| | 7 | 408000 | 40.8 |
| | 8 | 593000 | 59.3 |
| | 9 | 548000 | 54.8 |
| | 10 | 598000 | 59.8 |
| | 11 | 501000 | 50.1 |
| | 12 | 412000 | 41.2 |

# ANNEXURE - III

## Mean percentages of cell adhesion

| Groups | Samples | Cells/disc | % cell adhesion |
|---|---|---|---|
| G | 1 | 460000 | 46 |
| | 2 | 315000 | 31.5 |
| | 3 | 455000 | 45.5 |
| | 4 | 800000 | 80 |
| | 5 | 415000 | 41.5 |
| | 6 | 370000 | 37 |
| | 7 | 462000 | 46.2 |
| | 8 | 442000 | 44.2 |
| | 9 | 512000 | 51.2 |
| | 10 | 430000 | 43 |
| | 11 | 380000 | 38 |
| | 12 | 505000 | 50.5 |
| C | 1 | 405000 | 40.5 |
| | 2 | 421000 | 42.1 |
| | 3 | 575000 | 57.5 |
| | 4 | 484000 | 48.4 |
| | 5 | 475000 | 47.5 |
| | 6 | 510000 | 51 |
| | 7 | 532000 | 53.2 |
| | 8 | 493000 | 49.3 |
| | 9 | 512000 | 51.2 |
| | 10 | 455000 | 45.5 |
| | 11 | 545000 | 54.5 |
| | 12 | 502000 | 50.2 |

# ANNEXURE - IV

## Mean percentages of cell proliferation

| Groups | samples | OD | Mean | % cell viability |
|---|---|---|---|---|
| PC | | 0.409 | | |
| | | 0.407 | | |
| | 1 | 0.431 | 0.41567 | 34.4618 |
| | | 0.487 | | |
| | | 0.468 | | |
| | 2 | 0.461 | 0.472 | 39.1322 |
| | | 0.417 | | |
| | | 0.458 | | |
| | 3 | 0.45 | 0.44167 | 36.6174 |
| | | 0.576 | | |
| | | 0.581 | | |
| | 4 | 0.597 | 0.58467 | 48.4731 |
| | | 0.523 | | |
| | | 0.499 | | |
| | 5 | 0.574 | 0.532 | 44.1067 |
| | | 0.47 | | |
| | | 0.48 | | |
| | 6 | 0.489 | 0.47967 | 39.7679 |
| | | 0.427 | | |
| | | 0.447 | | |
| | 7 | 0.34 | 0.40467 | 33.5498 |
| | | 0.425 | | |
| | | 0.662 | | |
| | 8 | 0.602 | 0.563 | 46.6768 |
| | | 0.498 | | |
| | | 0.538 | | |
| | 9 | 0.591 | 0.54233 | 44.9634 |
| | | 0.481 | | |
| | | 0.491 | | |
| | 10 | 0.501 | 0.491 | 40.7075 |
| | | 0.421 | | |
| | | 0.415 | | |
| | 11 | 0.441 | 0.42567 | 35.2909 |
| | | 0.501 | | |
| | | 0.441 | | |
| | 12 | 0.467 | 0.46967 | 38.9388 |

# ANNEXURE - IV

## Mean percentages of cell proliferation

| Groups | samples | OD | Mean | % cell viability |
|---|---|---|---|---|
| NC | 1 | 0.832 | | |
| | 2 | 0.901 | | |
| | 3 | 0.939 | | |
| | 4 | 1.864 | | |
| | 5 | 0.857 | | |
| | 6 | 1.371 | | |
| | 7 | 1.129 | | |
| | 8 | 1.443 | | |
| | 9 | 1.245 | | |
| | 10 | 1.654 | | |
| | 11 | 0.941 | | |
| | 12 | 1.298 | 1.20617 | 100 |

# ANNEXURE - IV

## Mean percentages of cell proliferation

| Groups | samples | OD | Mean | % cell viability |
|---|---|---|---|---|
| G | 1 | 1.18 | 1.15833 | 96.0343 |
| | | 1.094 | | |
| | | 1.201 | | |
| | 2 | 1.229 | 1.141 | 94.5972 |
| | | 1.102 | | |
| | | 1.092 | | |
| | 3 | 1.279 | 1.18 | 97.8306 |
| | | 1.084 | | |
| | | 1.177 | | |
| | 4 | 0.871 | 0.87567 | 72.5991 |
| | | 0.843 | | |
| | | 0.913 | | |
| | 5 | 0.968 | 0.93933 | 77.8776 |
| | | 0.916 | | |
| | | 0.934 | | |
| | 6 | 0.963 | 0.93033 | 77.1314 |
| | | 0.862 | | |
| | | 0.966 | | |
| | 7 | 1.009 | 1.071 | 88.7937 |
| | | 1.102 | | |
| | | 1.102 | | |
| | 8 | 1.28 | 1.19167 | 98.7978 |
| | | 1.194 | | |
| | | 1.101 | | |
| | 9 | 1.168 | 1.106 | 91.6955 |
| | | 1.116 | | |
| | | 1.034 | | |
| | 10 | 1.113 | 1.11367 | 92.3311 |
| | | 1.162 | | |
| | | 1.066 | | |
| | 11 | 1.079 | 1.24667 | 103.358 |
| | | 1.284 | | |
| | | 1.377 | | |
| | 12 | 1.071 | 1.14233 | 94.7078 |
| | | 1.243 | | |
| | | 1.113 | | |

# ANNEXURE - IV

## Mean percentages of cell proliferation

| Groups | Samples | OD | Mean | % cell viability |
|---|---|---|---|---|
| C | | 1.069 | | |
| | | 1.082 | | |
| | 1 | 1.116 | 1.089 | 90.286 |
| | | 1.134 | | |
| | | 1.123 | | |
| | 2 | 1.12 | 1.12567 | 93.326 |
| | | 1.122 | | |
| | | 1.161 | | |
| | 3 | 1.154 | 1.14567 | 94.9841 |
| | | 1.106 | | |
| | | 1.063 | | |
| | 4 | 1.405 | 1.19133 | 98.7702 |
| | | 1.143 | | |
| | | 1.148 | | |
| | 5 | 1.125 | 1.13867 | 94.4038 |
| | | 1.135 | | |
| | | 1.146 | | |
| | 6 | 1.139 | 1.14 | 94.5143 |
| | | 1.109 | | |
| | | 1.132 | | |
| | 7 | 1.125 | 1.122 | 93.022 |
| | | 1.121 | | |
| | | 1.101 | | |
| | 8 | 1.166 | 1.12933 | 93.63 |
| | | 1.154 | | |
| | | 1.132 | | |
| | 9 | 1.156 | 1.14733 | 95.1223 |
| | | 1.144 | | |
| | | 1.132 | | |
| | 10 | 1.149 | 1.14167 | 94.6525 |
| | | 1.103 | | |
| | | 1.091 | | |
| | 11 | 1.136 | 1.11 | 92.0271 |
| | | 1.151 | | |
| | | 1.125 | | |
| | 12 | 1.132 | 1.136 | 94.1827 |